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**BIOLOGICAL SCIENCES  
DIVISION**

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**1991 PROGRAMS**

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**OFFICE OF NAVAL RESEARCH**  
**800 North Quincy Street**  
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## **INTRODUCTION**

The Biological Sciences Division is responsible for developing the fundamental knowledge that serves as the base for future Navy and Marine Corps needs. The division supports basic research in molecular biology, cell biology, molecular marine biology, immunophysiology, biochemistry, biophysics, molecular genetics, microbiology and sensory biology.

ONR research funds are divided, for management purposes, between two components: Core Programs and Accelerated Research Initiatives (ARI's). Core Programs draw on funds that remain relatively constant from year to year and can be used to support a variety of fundamental research activities of interest to ONR. ARI's on the other hand, exist for limited periods of time, usually five years, to address specific research needs that benefit from concentrated and innovative effort.

The Biological Sciences Division administers two programs, Molecular Biology and Systems Biology. In addition, we administer Education and Training programs. These programs are briefly described below.

### **Molecular Biology**

The primary naval concerns that provide a focus and rationale for the Molecular Biology Program are marine biofouling, biocorrosion, the need for novel materials and novel catalysts, requirements for highly sensitive and selective detector systems, and environmental quality. Guided by these naval concerns, the program emphasizes basic research activities that concentrate on molecular aspects of marine biology and on determining the principles of structure and function of biological molecules.

### **Systems Biology**

The objective of the Systems Biology Program is to support basic research on responses of complex organisms, particularly man, to unique features of naval environments. The focus is on systems and integrated responses involved in host defenses at the cellular and molecular levels. The primary goals of the Program are 1) a better understanding of the functional consequences of severe stress, particularly traumatic injury, and 2) determining the principles of biological sensors that can be translated into militarily useful devices.

Programs are grouped into three major area in Systems Biology. (1) Sensory Biophysics is directed at investigating membrane events involved in sensing environmental changes, particularly mechanisms of ion regulation and signal transduction. (2) Immunophysiology is directed at understanding the neural-immune interactions controlling the acute phase response in trauma and infection, and early repair processes in wound beds. (3) Cell Biology is directed at understanding cellular responses to injury and to hydrostatic pressures

encountered by the working diver.

### **Education and Training**

Training programs in Molecular and Systems Biology are intended to provide promising young scientists at the graduate and post-doctoral levels with state-of-the-art training in research areas outlined in this brochure. We also support education programs at historically black colleges and universities and minority schools. Our goal is to increase the number of minority students who choose science and engineering for a profession. Thus, we support efforts to prepare undergraduates for graduate study in the biological sciences at major research institutions. Education programs supported by us typically involve recruiting and retention efforts, curriculum enhancement, and undergraduate research programs.

### **ORGANIZATION OF PROGRAM SUMMARY**

On the following pages, each research project is summarized in a description written by the principal investigator. In many cases, the description has been edited by a scientific officer at ONR to conform to a two page limitation and to provide consistency of presentation. The summaries are organized by research program and assembled alphabetically by PI.

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## **BIODETERIORATION**

### **CORE PROGRAM**

**SCIENTIFIC OFFICERS: DR. RANDALL S. ALBERTE, DR. ERIC EISENSTADT,  
AND DR. STEVEN SNYDER**

**PROGRAM OBJECTIVE: TO DETERMINE THE MOLECULAR MECHANISMS  
RESPONSIBLE FOR BIOFOULING AND BIOCORROSION IN MARINE ENVIRONMENTS.**

**NAVY OBJECTIVE: TO DEVELOP TECHNOLOGIES FOR ADDRESSING PROBLEMS OF  
BIOFOULING AND BIOCORROSION IN THE MARINE ENVIRONMENT.**

**BIOCATALYSIS IN NON-AQUEOUS SOLVENTS**

**CORE PROGRAM**

**SCIENTIFIC OFFICER: DR. HAROLD J. BRIGHT**

**PROGRAM OBJECTIVE:** TO DESIGN ENZYMES TO BE SOLUBLE, STABLE, AND HIGHLY ACTIVE IN NON-AQUEOUS SOLVENTS.

**NAVY OBJECTIVE:** TO INTRODUCE BIOCATALYSTS TO THE ARENA OF ORGANIC CHEMISTRY AND THEREBY FACILITATE EXISTING AND POTENTIAL PROCESSES FOR THE CONSTRUCTION OF NOVEL AND NAVY-RELEVANT MATERIALS.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-91-J-1397

R&T CODE: 4412098

PRINCIPAL INVESTIGATOR: Frances H. Arnold

INSTITUTION: California Institute of Technology

GRANT TITLE: Enzyme Engineering for Nonaqueous Solvents: Mutagenesis of  $\alpha$ -Lytic Protease

REPORTING PERIOD: 1 May 1991 - 1 June 1991

AWARD PERIOD: 1 February 1991 - 31 January 1995

OBJECTIVE: To demonstrate that enzymes can be engineered at the level of their amino acid sequences to improve their stability and catalytic activity in polar nonaqueous solvents

### ACCOMPLISHMENTS:

We are working with a  $\alpha$ -lytic protease, a highly stable serine protease containing 198 amino acids and three disulfide bonds, as a model enzyme for use in organic media. In organic solvents,  $\alpha$ -lytic and other proteases are potentially useful for peptide synthesis by aminolysis of peptide esters or by reversal of the normal hydrolysis reaction. A convenient mutagenesis and expression system for  $\alpha$ -lytic protease has been developed.  $\alpha$ -lytic protease was expressed in *E. coli* from a synthetic gene (obtained from Prof. John Richards, Caltech). G or C found at the third codon position are replaced by A or T in this gene, which was designed for cassette mutagenesis. The gene was inserted into an  $\alpha$ -lytic expression vector (pALP5) kindly supplied by Prof. D. Agard, UCSF, which includes the *PhoA* promoter, the first 18 amino acids of the  $\alpha$ -lytic presequence, the entire  $\alpha$ -lytic prosequence, and the native gene coding for the mature enzyme. In the resulting hybrid construct which contains the synthetic gene, the first 87 bases coding for the mature enzyme are from the native  $\alpha$ -lytic protease gene.

We have proposed and tested a strategy for creating a nonaqueous solvent-stable enzymes that includes rendering the protein surface more hydrophobic. To study the effects of substituting surface charged amino acid with neutral ones, we randomly altered the sequence of  $\alpha$ -lytic protease at two charged sites, Arg 45 and Arg 78. At each position we have found several variants whose stability is significantly greater than that of the wild-type enzyme in 84% DMF. In six of the seven variants which exhibit improved stability, the charge has been replaced by a neutral or hydrophobic amino acid. A mutant with two surface charge substitutions is 27 times more stable than wild-type in 84% DMF. A paper describing this research has been submitted for publication (Martinez and Arnold).

We have also begun to tackle the problem of improving enzyme activity in nonaqueous solvents. Due to our relatively poor understanding of the effects of amino acid substitutions on activity, we have chosen to use random mutagenesis by a PCR technique, combined with an efficient screening procedure, to identify mutations that increase catalytic activity in non-aqueous solvents. Sequencing and characterization of such mutants will aid us in determining the important factors influencing activity in nonaqueous solvents. The catalytic site of  $\alpha$ -lytic protease is surrounded by five stretches of polypeptide chain, encompassing amino acid positions 101-104, 136-142, 145-147, 158-162, and 179-182. To limit the region of mutagenesis to those positions near the active site and most likely to affect catalysis, PCR-based random mutagenesis will affect a 230 bp *NsiI*-*NcoI* DNF fragment that covers the last four stretches. A larger PCR-

amplified region (450 bp, spanning the Nsi-NcoI fragment) will be digested with NsiI and NcoI, after which the cassette will be inserted into the expression vector for expression in E. coli. The modified PCR technique used for random mutagenesis has already been used successfully in this laboratory for mutagenesis of subtilisin E (Chen and Arnold, submitted for publication).

Following transformation of the E. coli with the randomly-mutated expression vector, the bacteria are plated on agar dishes and then screened for activity in aqueous medium and in the presence of dimethylformamide. We are currently working out the details of this screening procedure.

#### SIGNIFICANCE:

In the last twenty years the use of enzymes in industry has expanded rapidly. The reasons are straightforward: enzymes are cost-effective, convenient, precise, and efficient. However, a major drawback to the use of enzymes in chemical processing is their low stability in polar organic solvents. Organic solvents have many advantages over water as a reaction medium for industrial processes. These include, but are not limited to, increased ease of product recovery, enhanced solubility of hydrophobic substrates, and reduced microbial contamination of biocatalytic reactors. The use of biocatalysts in large scale chemical production is also highly desirable. Enzymes function under mild conditions (room temperature and pressure), have very high turnover numbers (which far exceed the turnover numbers for most commercial catalysts), and most importantly are highly specific. The degree of specificity of an enzyme is such that it can convert a complex reactant to produce with no-side reactions, enabling simple downstream processing of the reaction mixture. Consequently, enzymatic catalysis in organic solvents holds the promise of revolutionizing the modern industrial approach to production of specialty and perhaps, eventually, even bulk chemicals. This research should provide us with clearly defined strategies for selecting enzymes that are suitable for use in polar organic solvents and for engineering enzymes to exhibit improved stability and activity in these solvents.

#### PUBLICATIONS AND REPORTS (last 12 months):

"Replacement of surface charged residues increases the stability of  $\alpha$ -lytic protease in organic solvents", P. Martinez and F.H. Arnold, submitted.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-91-J-1230

R&T CODE: 4412091

PRINCIPAL INVESTIGATOR: Daniel E. Kahne

INSTITUTION: Princeton University

GRANT TITLE: Studies Directed Towards the Solubilization of Peptides and Proteins in Organic Solvents

REPORTING PERIOD: 1 November 1990 - 1 June 1991

AWARD PERIOD: 1 November 1990 - 31 October 1993

OBJECTIVE: Our objective is to develop a class of small amphiphilic molecules that interact with membranes and which can be used to alter the solubility properties of attached peptides and proteins. The longterm goal is to be able to facilitate the transport of hydrophilic molecules into or across organic environments.

ACCOMPLISHMENTS (last 8 months): During the past eight months we have completed the synthesis of our first generation of amphiphilic molecules. Six steroidal glycoconjugates were constructed by attaching sugars to various cholic acid derivatives using a glycosylation method developed in our laboratory. By varying the conditions of the glycosylation reaction we were able to obtain either an  $\alpha\alpha$  or a  $\beta\beta$  bis glycosylated steroid stereospecifically. We are investigating how the stereochemistry of the glycosidic linkages affects the packing of these steroidal glycoconjugates against one another or against other surfaces. We also developed an efficient synthetic sequence to convert the steroid A-B ring junction from cis to trans. This transformation changes the overall shape of the molecule from a curved surface (cis A-B) to a flatter surface (trans A-B). Finally, we have developed chemistry to introduce an amine at C3 into these systems. This will allow us to evaluate the effect that charge has on the properties of the compounds.

We have measured the absolute solubility of these steroidal glycoconjugates in octanol and water as well as their partition coefficients between octanol and water. Compared to cholic acid methyl ester, all the glycosylated steroids are more water soluble by several order of magnitude (at least  $10^3$  to  $10^4$ ). At the same time, however, the glycosylated steroids are also more soluble than cholic methyl ester in organic solvents. We have thus increased the amphiphilicity of the steroidal conjugates as planned. There are some interesting differences in solubility between the steroidal glycosides. The  $\beta$  anomers are always more water soluble than the  $\alpha$  anomers. Interestingly, the cis  $\beta\beta$



compound is also infinitely soluble in octanol (it forms a gel at high concentrations).

We have also developed an assay to measure the transport rates of molecules across model membranes. We have found that the cis  $\alpha\alpha$  compound facilitates the transport of a hydrophilic molecule (AZT) across model membranes. The increased rate of transport is catalyzed by some as yet uncharacterized noncovalent interactions between the amphiphilic surface and the hydrophilic AZT molecule.

SIGNIFICANCE: Developing a strategy to alter the surface properties of hydrophilic peptides and proteins so that they partition directly into lipophilic environments would be very useful. It would solve the problem of how to get hydrophilic peptides to cross membrane boundaries. It might also allow one to explore in greater detail chemistry that takes place in membranes. For example, if one could design peptide models for ion channels that stably insert into membranes, one could begin to test theories about ion channel specificity and structure on relatively simple systems. This in turn might lead to the design of small systems which catalyze the transport of ions across membranes. One might also be able to anchor proteins at interfaces if portions of the protein can be made to insert into membranes.

WORK PLAN: The objectives next year are to:

- 1) Determine how the steroidal glycoconjugates interact with membranes - i.e., insertion vs. binding at the surface.
- 2) Determine whether the steroidal glycoconjugates alter the permeability properties of membranes. If they do, we will try to determine whether they act as detergents, channel, or carriers.
- 3) Use our transport assay to measure the transport rates of each steroidal glycoconjugate across model membranes.
- 4) Attach promising glycoconjugates to hydrophilic peptides and assay whether the transport rates across model membranes and the absolute solubility in hydrophobic solvents is altered.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-900J-4002

R&T CODE: 400x079

PRINCIPAL INVESTIGATOR: Kenneth M. Merz Jr.

INSTITUTION: The Pennsylvania State University

GRANT TITLE: Non-Aqueous Dynamics of Proteins

REPORTING PERIOD: 1 July 1990 - 30 June 1991

AWARD PERIOD: 1 July 1990 - 30 June 1993

OBJECTIVE: To investigate how protein structure, function, and dynamics is affected by non-aqueous solvents using computer simulation. This information can then be used to assist in the design of proteins with the desired stability and function in a non-aqueous environment.

ACCOMPLISHMENTS (last 12 months): We have developed software that allows us to study proteins in any solvent system we choose. There were some difficulties in accomplishing this, but we have overcome them and now have working software. We initially planned on studying subtilisin in hexane, but we decided to try a smaller protein first. This decision was made in order to first test our software on a smaller system and to be able to carry out much longer simulations. We decided to first study bovine pancreatic trypsin inhibitor (BPTI) in water and in chloroform. Hexane was not used because of technical problems with implementing this solvent in an MD program. We are continuing to try to solve this problem. The BPTI study is nearly completed, but some preliminary results are quite striking. The simulations suggest that BPTI in chloroform undergoes an initial rapid conformational change, which mostly involves burying charged surface amino acids into the surface of the protein. Once this is done the motion of BPTI is severely restricted relative to the water simulation. This rigidity fits nicely into the ideas put forward by Klibanov and co-workers where they suggest and provide evidence for protein rigidity in non-aqueous solvents. We have also been studying the antibiotic ionophores valinomycin and nonactin in non-aqueous solvents. The work is nearing completion and a more thorough description of the work will be given in the next Progress Report.

SIGNIFICANCE: Elucidating molecular-level details regarding how a protein responds to a non-aqueous environment has numerous bioengineering applications. This information can be used, for example, to design enzymes of enhanced stability and altered function to non-aqueous solvents. This research should also provide fundamental insights into the solvation of proteins.

WORK PLAN (next 12 months): We plan to continue our study of the BPTI trajectories as well as the valinomycin and nonactin studies. We expect to complete this work in the next months. We plan on writing a grant for supercomputer time from the Pittsburgh Supercomputer Center to carry out MD simulations of subtilisin in non-aqueous solvents. Wong and co-workers have developed subtilisin mutants that are more stable in non-aqueous solvents than is the native protein. We would like to study both Wong's mutants and the native protein in order to understand at the molecular level what are the important factors involved in stabilizing the mutant protein relative to the native form. Finally, we plan to start studying the binding affinity of various substrates to chymotrypsin in both aqueous and non-aqueous environments.

ANNUAL PROGRESS REPORT

GRANT #: N00014-91-J-1652

R&amp;T CODE: 4412108---01

PRINCIPAL INVESTIGATOR: Chi-Huey WongINSTITUTION: The Scripps Research InstituteGRANT TITLE: Designing Enzymes for Homogeneous Catalysis in Organic SolventREPORTING PERIOD: 1 April 1991 - 30 May 1991 (2 months)AWARD PERIOD: 1 April 1991 - 31 March 1994OBJECTIVE: To develop a stable and active subtilisin variant for homogeneous catalysis in organic solvents.

ACCOMPLISHMENTS (last 1 month): The enzyme subtilisin 8397 variant developed in our laboratories was active and stable in dimethylformamide containing 10% aqueous solution. This variant prepared via site-directed mutagenesis contains 5 point mutations designed to enhance the protein's internal H-bonding and  $\text{Ca}^{++}$ -binding interactions, Van der Waal's contact, and conformational restriction, and minimize the surface charge. The mutations are Met50phe, Gly169Ala, Asn76Asp, Gln206Cys and Asn218Ser. This variant is about 10,000 times more stable and 5 times more active than the wild-type enzyme in aqueous solution and in dimethylformamide. The half-life in anhydrous dimethylformamide is about 20 days and in 10% dimethylformamide is more than 40 days. This enzyme has been used as a catalyst in regioselective acylation of polyhydroxylated compounds including sugars and nucleosides, enantioselective transesterification and hydrolysis of chiral alcohols, acids and amino acids, polymerization of amino acid and peptide esters, and segment coupling of peptides. Further chemical modification of the variant enzyme via selective modification of the active site serine residue to cysteine converted the enzyme to a highly effective peptide ligase for aminolysis of peptide segment in organic solvents and in aqueous solutions, with no detectable secondary peptide hydrolysis or transamidation.

SIGNIFICANCE: Investigation of the effects of amino acid side chains of subtilisin on the enzyme's stability and catalysis in dimethylformamide via site-directed mutagenesis and chemical modification provides a new opportunity for the development of guiding principles for use to design enzymes for reactions in organic solvents.

WORK PLAN (next 12 months): The objective next year is to characterize the modified variant containing an active-site

cysteine and to determine the kinetic parameters. Evaluation of the catalyst for homogeneous catalysis in organic solvents will be carried out. Site-directed mutagenesis of 8397 to replace the surface residue Lys-256 to Tyr and Lys-43 to Asn will be conducted to further minimize the surface charge. These mutations are expected to create a new variant which may be active and soluble in a higher concentration of organic solvents.

ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 441z016

PRINCIPAL INVESTIGATOR: Gerald Bohlander

INSTITUTION: David Taylor Research Center Detachment

GRANT TITLE: Marine Biofilm Sampling Program

REPORTING PERIOD: 1 June 1991 - 1 July 1991

AWARD PERIOD: 1 June 1991 - 30 September 1991

OBJECTIVE: Funds are provided to sample the biofilm composition of Naval platforms at four ports representative of the diverse fouling communities that are encountered by Naval vessels. Sampling will be performed at three depths; waterline, maximum beam and keel, and at four locations on both port and starboard sides. The samples collected will be fixed in a manner that will permit detailed analysis of the microfouling composition of the biofilms using standard laboratory techniques.

ACCOMPLISHMENTS: This is a new grant.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-88-K-0158

R&T CODE: 4412035

PRINCIPAL INVESTIGATOR: Robert P. Burchard

INSTITUTION: University of Maryland, Baltimore County

GRANT TITLE: Inter-species Inhibition of Adhesion Between  
Gliding Bacteria from Marine

REPORT PERIOD: 1 July 90 - 30 May 1991

AWARD PERIOD: 1 Jan 88 - 30 May 91

### OBJECTIVE:

1. To characterize the extracellular slime of selected marine gliding bacteria;
2. To determine the function(s) of extracellular slime in adhesion and motility of these gliding bacteria; and
3. To characterize the cell surface adhesins of selected marine gliding bacteria and how they interact with inhibitors of adhesion.

### ACCOMPLISHMENTS:

1. The polysaccharidic component of the extracellular slime of Flexibacter maritimus is predominantly a glucose polymer. In collaboration with Ian Sutherland (Edinburgh), linkage and branching is being determined. A second heteropolysaccharide (D-glucose, D-galactose, D-mannose and L-rhamnose) has also been identified.

2. The slime of Cytophaga sp. Strain 1327B has proven more amenable to analysis of F. maritimus since it is released into the extracellular medium. It has been partially purified by gel filtration. Preliminary evidence suggests that the viscous properties of the slime are due to the presence of polypeptide(s), not polysaccharide as predicted. W.H. Schwarz (John Hopkins) has performed rheological analysis of this slime using a Weissenberg rheogoniometer. Specifically, values of the apparent shear viscosity versus the rate-of-strain, and the complex modulus versus the frequency of oscillation were obtained. Preliminary data indicate that the slime is non-Newtonian and viscoelastic. These results will be applied to a mathematical model to describe motility and adhesion of some gliding bacteria.

3. We have extended our studies of the surface properties of a variety of gliding bacteria, some grown under different conditions. The intent has been to relate cell surface hydrophobicity to adhesion. Hydrophobicity has been assayed by bacterial adherence to hydrocarbons (BATH), hydrophobic interaction chromatography (HIC) and salt aggregation (SAT). No generalization about the surfaces of these bacteria can be reached nor is there any one measure of hydrophobicity that is predictive of adhesion capability.

4. The bacterial adhesion inhibitory factor (AIF) produced by a marine biofilm gliding bacterium has been at least partially purified by gel exclusion and anion exchange chromatography. It appears to be a high molecular polysaccharide which is sensitive to glucuronidase from E. coli. The material inhibits adhesion by a variety of aquatic bacteria, (including both gliders and non-gliders from natural and industrial biofilms) to a variety of substrata differing widely in critical surface energy (CSE). It is most effective on high CSE substrata. We are currently working to improve the efficiency of recovery of AIF in order to generate enough material to permit its identification.

**SIGNIFICANCE:**

\* Characterization of the production, structure and rheology of the extracellular slime of gliding bacteria should lead to an understanding of its role in their adhesion and motility.

\* Studies of the hydrophobicity of various gliding bacteria and of their adhesion to and motility on substrata differing in critical surface energy should lead to an understanding of the mechanism(s) of adhesion of these bacteria.

\* An immobilized radiolabelling technique has provided a tool for the identification of surface proteins, including putative adhesins, that make contact with substrata. Application of this technique permits molecular characterization of the cell surfaces of adhesion-defective mutants and of bacteria grown under conditions resulting in differences in cell surface polymer production. It also provides a powerful taxonomic tool for the gliding bacteria.

\* A unique type of inter-specific inhibition of bacterial adhesion within biofilms may have been discovered. The inhibitor, tentatively identified as an exopolysaccharide, may represent a new class of anti-fouling agent.

**WORK PLAN (next 12 months):** Identification of cell surface-exposed polypeptides of several gliding bacteria in collaboration with R.A. Bloodgood (U.Va) are ongoing. This research utilizes a surface-immobilized iodination catalyst (Indo-Gen).

A) We are determining whether extracellular polymers mask the bacterial surface, preventing cell envelope proteins from contacting substrata.

B) We are determining whether AIF, the adhesion inhibitor described above, interferes with labelling of cell surface proteins.

C) We previously identified one predominant ~42 kDa polypeptide exposed on the surface of Cytophaga RB1058, the marine biofilm isolate that led to the AIF research. We hypothesize that this polypeptide is the adhesin of this bacterium. Several adhesion-defective mutants and revertants have been isolated. We are currently comparing the surface-exposed polypeptide(s) of these strains with those of the wild-type.

D) We are completing studies that demonstrate that arrays of surface-exposed proteins can be used for bacterial taxonomy (i.e. closely related bacteria have similar arrays of surface polypeptides).



## ANNUAL PROGRESS REPORT

Grant # N00014-91-J-1108

R & T Project 4412089---01

Principal Investigators: Kyle D. Hoagland; Michael R. Gretz

Institutions: University of Nebraska; George Mason University

Grant Title: "Biochemistry of Fouling Marine Diatom Adhesives and the Effects of Substrate Preconditioning on Adhesion"

Reporting Period: December 1990 - May 1991 (six months)

Award Period: December 1990 - September 1993

**Objectives:** 1) Isolate and mass culture diatoms involved in biofouling, develop methods to mechanically purify adhesive extracellular polymeric substances (EPS) from these diatoms and chemically characterize these polymers primarily using modern carbohydrate analysis techniques; 2) Determine the composition of marine substrate precoatings; 3) Describe the sequence of diatom adhesion events, and; 4) Determine the effects of substrate preconditioning on diatom adhesion.

**Accomplishments (six months):** As a prelude to our investigations of diatom biofouling phenomena we have completed a comprehensive review of diatom extracellular polymeric substance fine structure, chemistry and physiology (submitted to the *Journal of Phycology* [attached]). We have agreed upon a PC based reference management system and are creating a diatom/biofouling database incorporating our collections and references culled from Current Contents on Disk, Biosis, Chem Abstracts, etc.

Significant progress has been made in the first six months of the project with respect to culturing diatoms and preparing purified stalk fractions for biochemical analyses. Work to date has focused on two stalk-forming species: the marine biofouling diatom *Achnanthes longipes* and the freshwater diatom *Cymbella cistula*. Two clones of each of these diatoms are in mass culture for the first time, including one isolate of *A. longipes* from the NEIS collection in Japan (isolate 330) and one new isolate from Virginia sea shores, as well as one new isolate of *C. cistula* from Oregon and one previous clone from a Nebraska reservoir. In addition, four clones of the marine fouling, capsule-forming diatom *Amphora coffeaeformis* (Bigelow 211M, UTEX 2038, 2080, and 2036), two additional freshwater species of *Cymbella* (*mexicana* and *triangulum*), and one clone of the marine fouling, stalk-forming diatom *Licmophora* are presently in large scale culture. All cultures are being maintained at 16°C under a 12:12 light:dark cycle, on a continuous shaker and/or in static culture. Marine diatoms are cultured in two types of marine media to determine optimal conditions for mucilage production.

Large quantities of purified stalk fractions have been successfully prepared from clonal cultures of *A. longipes* and *C. cistula* by physical separation. Stalk fractions are prepared by shearing cells from stalks by tissue grinding at high speed for ten minutes, filtering the cell-stalk mixture through 65-µm mesh Nitex netting, and freeze-drying the stalks retained on the netting. All stalk fractions are kept on ice during preparation to prevent degradation of the constituent polymers.

Stalk material from cultured diatoms, isolated as above, is remarkably free of contaminants (protoplasmic and otherwise). This greatly simplifies our approach for biochemical characterization. Because we have only small quantities of this highly pure material, we have chosen to first characterize the polysaccharide components. Hydrolysis of *Achnanthes* and *Cymbella* stalks followed by standard techniques for monosaccharide analysis shows a predominance of fucose, glucose and galactose in stalks

from *Achnanthes* whereas the freshwater species *Cymbella* contains mainly galactose, xylose and glucose. A relationship between the quantity of deoxy-hexose in diatom polysaccharides and the salinity of habitat has been reported previously. We are examining differences in polymer chemistry/mechanisms of attachment between diatoms that reside in freshwater and some marine forms in hopes that this may yield insight into potential structural/functional relationships.

Methylation analysis of *Achnanthes* and *Cymbella* stalks has given the data summarized in Table 1. We have now begun routinely using a new combination of methylation analysis techniques that has shortened our methylation, purification and derivatization time considerably. One of the more significant of these is the substitution of a two step butyllithium-methyl iodide methylation for our old standby methylsulfinyl carbanion-methyl iodide method. We have now used our "improved" techniques on a wide variety of polysaccharide samples with excellent, reproducible results and are actively developing techniques to optimize methylation analysis of sulfated and anhydro-sugar containing polymers.

Table 1. Glycosyl linkage composition expressed as the position(s) of substitution in addition to C-1 (i.e. 4-hexose = 1,4,5-tri-O-acetyl-1-deuterio-2,3,6-tri-O-methylhexitol and t-hexose = 1,5 di-O-acetyl-1-deuterio-2,3,4,6-tetra-O-methylhexitol), determined from per-O-methylated alditol acetates from mechanically isolated stalks. Only major linkages are reported, based on peak area and published response factors.

Species	Fuc	Gal	Glc	Xyl
<i>Achnanthes longipes</i>	3- t-		4-	2,3,4- <sub>p</sub> <sup>1</sup> or 2,3,5- <sub>f</sub> 4- <sub>p</sub> or 5- <sub>f</sub>
<i>Cymbella cistula</i>		4,6- 3,4,6- 3,4- 4-	4-	4- <sub>p</sub> or 5- <sub>f</sub> t-

<sup>1</sup> subscript p = pyranose, f = furanose; not distinguishable with methylation analysis.

Partial fractionation and purification of methylated polymers preceding the reduction and acetylation steps can provide some information as to the anionic character of the polymers. Preliminary results indicate that the *Achnanthes* polymer containing 2,3,4-linked-xylopyranosyl or 2,3,5-linked-xylofuranosyl residues may be sulfated as also may be the putative xylogalactan from *Cymbella*. The 4-linked glucosyl residues from both species were present in a non-charged fraction which may indicate the presence of cellulose. No uronic acids were detected in our analyses. It would appear that the stalks from these two species are quite distinct with respect to their polysaccharide chemistry. Substantiation of the above speculation is currently underway using X-ray, NMR and spectrophotometric methods.

**Significance:** The development of methods for mass culture of fouling diatoms and mechanical isolation and characterization of adhesive EPS components provides information necessary to begin modeling the diatom-substrate interaction at the molecular level. Details of the chemistry of polymers involved in diatom biofouling provide valuable information necessary for studies of biosynthesis and genetics of attachment phenomena.

#### Publications supported by ONR (six months):

1. A review paper was submitted:

Diatom extracellular polymer substances: fine structure, chemistry, physiology and function. Kyle A. Hoagland, James R. Rosowski, Michael R. Gretz & Stephen C. Roemer. *Journal of Phycology* (in review 1991)

### THREE MONTH PROGRESS REPORT

Grant #: N00014

R&T Code: 4412206

PRINCIPAL INVESTIGATOR: Joanne M. Jones

INSTITUTION: NAVSWC/WO, Code R301

GRANT TITLE: Role and Regulation of Emulsifier Production in  
Microbial Degradation of Heavy Hydrocarbons

REPORTING PERIOD: 12 March 1991- 3 June 1991

AWARD PERIOD: 29 Feb 1991- 30 Sept 1991

OBJECTIVE: To provide information about the mechanisms of heavy hydrocarbon degradation by mixed communities of bacteria and how the microorganisms in those communities interact with one another and with the hydrocarbon molecules through regulation of production and degradation of emulsifiers.

ACCOMPLISHMENTS (last 3 months): I have hired a post doctoral research fellow (Dr. Tammy Davidson) who has had extensive experience in isolation/purification of natural products while doing a post doctoral fellowship with Dr. Fred Singleton (COMB). Large quantities of the bioemulsifier from heavy hydrocarbon-degraders (Acinetobacter calcoaceticus NAV2 and RAG-1, Pseudomonas aeruginosa NAV6) have been prepared. These isolates are able to grow and produce emulsifiers on Jet-A fuel, JP-5 fuel, motor oil and polyurethane paint as the sole carbon source. The emulsifier is being concentrated by several methods (i.e., lyophilization, ammonium sulfate, etc). The lyophilized cell-free emulsifier has been fractionated on a Sephadex G-50 column and the emulsifier activity is in the fraction containing protein as expected from previous work. Cell-associated and cell-free emulsifier preparations will be used to generate polyclonal antibody preparations. The antibody work (polyclonal, fluorescently-labeled antibodies and possible monoclonal antibody preparations) will be done in Dr. Vasta's laboratory at COMB.

Various conjugative plasmids, transposons and mobilizable suicide transposon donors have been requested and/or received from several sources for use in the identification of genes responsible for bioemulsifier production. The strains requested so far are being supplied from the Michigan Biotechnology Institute, COMB, Univ. of TN, Univ. of New South Wales (Australia) and Univ. of Postfach (Germany).

SIGNIFICANCE: The Navy has at least 6,000 underground leaking fuel tanks that could cost hundreds of billions of dollars to clean up using traditional physical/chemical treatment methods. The Navy has about 50 on-shore oil spills per year which average 4,000 gallons each with clean-up costs estimated

at \$7-\$10 million per year. Biosurfactants could be used to remove contaminants from soil particles and the contaminated groundwater could be pumped to the surface for treatment in an above-the-ground reactor. This type of bioremediation is called biostimulation and has been used successfully in the southeastern U.S. on solvent spills that included toluene, xylenes, phenol, methyl ethyl ketone and other solvents in the 100's of mg/l range. Bioslurry reactors are suited for highly contaminated (>1%) soils and could also use surfactants to flush contaminants from soil particles. Above-the-ground bioremediation would be more economical, easier to control and monitor the degradation processes compared to in situ biodegradation.

WORK PLAN (next 12 months): The cell-free and cell-associated forms of the emulsifiers will be used to make polyclonal antibodies. Fluorescently-labeled antibody preparations will be used to identify various mutant phenotypes for the bioemulsifier (i.e., mutants which make decreased amounts of emulsifier, mutants which are over-producers of emulsifier, changes in various components of the lipid-polysaccharide-protein complex of the emulsifier, etc).

Using transposon mutagenesis, we will be screening for a variety of mutant phenotypes including non-adherent and emulsan-deficient (will be more hydrophobic compared to cells with emulsifier capsule). We are interested in mutants that accumulate emulsifier but do not release the cell-associated material, that are over-producers of emulsifier and that have alteration(s) in the distribution or structure of the emulsifier (gradations of emulsifying properties). Fluorescently-labeled antibodies and fluorescently-labeled hydrocarbons will be used.

Emulsifier preparations will be used to coat heavy hydrocarbon substrates to determine if the emulsifier-treated hydrocarbons are more recalcitrant to biodegradation. Dr. George Pickwell (NOSC/Code 521, San Diego, CA) is currently testing the ability of biosurfactants from RAG-1 and other bacteria for their ability to wash weathered JP-5 fuel from soils by desorption and/or emulsification. The goal is for the contaminated groundwater to be pumped to the surface and treated in an above-the-ground bioreactor. Emulsan-coated Prudhoe Bay crude oil was shown by Foght et al. (1989) to be biodegraded at a slower rates than the original, untreated oil. Shosham and Rosenberg (1983) reported an emulsan-degrading bacteria (extracellular emulsan depolymerase = endoglycosidase) which solubilized emulsan as a source of carbon and energy. The use of surfactants to flush contaminants from soils and/or groundwater may require the degradation of the emulsifier followed by the degradation of the contaminants.

PUBLICATIONS AND REPORTS (last 3 months): None

# ANNUAL PROGRESS REPORT

GRANT #: N00014-87-K-0108

R&T CODE: 441h001

PRINCIPAL INVESTIAGTOR: David L. Kirchman

INSTITUTION: University of Delaware

GRANT TITLE: Regulation of Attached Bacterial Growth by Adsorbed Proteins

REPORTING PERIOD: 30 June 1990 - 31 May 1991

AWARD PERIOD: 1 May 1990 - 30 April 1992

OBJECTIVE: To examine interactions among adsorbed protein, bacteria, and bacterial attachment proteins.

ACCOMPLISH 1ENTS: Over the past year, we published our work showing how degradation of adsorbed protein varied with different surfaces and how that affected growth rates of attached bacteria. Briefly, percent degradation of the adsorbed protein by the marine bacterium Pseudomonas S9 was greater on high than on low energy surfaces. For cells that have been attached to surfaces for <4 h, growth rates were low on hydrophobic (low energy) surfaces and increased with increasing surface energy.

We next tried to determine the relative importance of adsorbed protein for attached assemblages of natural bacteria in actual seawater. But these experiments were not very satisfying, and it became apparent that we needed more information about the extracellular proteins in seawater.

We first tested vapor phase hydrogen (VPH) for measuring dissolved combined amino acids (DCAA) in seawater, which includes proteins and amino acids, for example, complexed with other macromolecules. VPH revealed up to 3-fold higher concentrations than found by the old method. The old method did detect non-protein DCAA. <10% of the extracellular, dissolved protein in seawater was equivalent to freshly-formed protein. The other 90% of the dissolved protein was functionally equivalent (in our bioassay) to melanoidin, which is protein with a glucose covalently-linked to  $\epsilon$ -amino groups of proteins (Keil 1991).

During work on bacterial attachment, we became interested in the general question of whether marine bacteria have specific mechanisms (like lectins) for attaching to specific surfaces, such as chitin. We have accumulated evidence that some marine bacteria use chitin specific attachment mechanisms related to bacterial nutrition: 1) there is a correlation between attachment to chitin and chitinase activity; 2) the trimer of chitin (tri-N-acetylglucosamine) specifically stimulates attachment; 3) attachment by a chitinase-overproducing mutant of Vibrio harveyi to chitin is greater than attachment by the wild-type, but the to strains do not differ in attachment to other surfaces; 4) a mild detergent extract of the bacterium followed by affinity chromatography yields a fraction with two polypeptides which inhibit attachment to chitin but to cellulose; 5) polyclonal antibodies raised against excreted chitinase, which cross-react with the "attachment polypeptides", inhibit bacterial attachment to chitin.

Our data suggest the following hypothesis: V. harveyi uses a polypeptide (perhaps two), with no chitinase activity, to adhere to chitin, and separate enzymes (chitinase and chitobiase) to actually degrade the surface.

SIGNIFICANCE: To examine interactions between the adsorbed organic film

and attached bacteria, we need information about the chemical structure of the film and of the bacterial attachment mechanisms. Our work suggests that protein in seawater, while reactive in common protein assays, has been modified by geochemical reactions and is no longer "recognized" as protein by bacterial proteases. The second theme of our work, specific attachment mediated by membrane polypeptides, should help us begin to understand the molecular mechanisms by which marine bacteria adhere to surfaces.

WORK PLAN (next 12 months): We have developed an HPLC assay to measure the geochemically-modified proteins in seawater, but the assay needs further work. Specifically, we need to confirm by NMR or mass spectrometry our hypothesized melanoidan structure. In our experiments on specific bacterial attachment, we have begun to examine the regulation of expression of the attachment polypeptide. To aid in this, we hope to sequence the N terminus of this polypeptide and develop specific attachment mutants.

PUBLICATIONS AND REPORTS:

Montgomery, M.T., N.W. Welschmeyer, and D.L. Kirchman. 1990. A simple and rapid assay for chitin: Application to sediment trap samples from the Subarctic Pacific. *Mar. Ecol. Prog. Ser.* 64: 301-308.

Nagata, T. and D.L. Kirchman. 1990. Filtration-induced release of dissolved free amino acids: application to cultures of marine protozoa. *Mar. Ecol. Prog. Ser.* 68: 1-5.

Samuelsson, M-O. and D.L. Kirchman. 1990. Relationship between surface hydrophobicity and the degradation of adsorbed protein by attached bacteria. *Appl. Environ. Microbiol.* 56: 3643-3648.

Keil, R.G. and D.L. Kirchman. Dissolved combined amino acids as estimated by vapor-phase hydrolysis method. *Mar. Chem.* In press.

Nagata, T. and D.L. Kirchman. Release of dissolved free and combined amino acids by bacterioivorous marine flagellates. *Limnol. Oceanogr.* In press.

Logan, B.E. and D.L. Kirchman. Increased uptake of dissolved organics by marine bacteria as a function of fluid motion. *Mar. Biol.* In press.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-WK24143

R&T CODE: 4412075

PRINCIPAL INVESTIGATOR: Michael B. McNeil

INSTITUTION: Naval Coastal Systems Center

GRANT TITLE: Mineralogical Fingerprints

REPORT PERIOD: 1 October 1990 - 30 June 1991

AWARD PERIOD: 1 October 1990 - 30 September 1991

**OBJECTIVE:** The objectives of this research program are to ascertain whether sulfiding corrosion of copper alloys induced by the action of bacterial suites containing sulfate-reducing bacteria produces corrosion products different mineralogically from the products of abiotic or microbiologically intermediated sulfiding corrosion, to identify other corrosion products distinctively characteristic of MC.

### ACCOMPLISHMENTS (last 9 months):

1. Specimens of 99 Cu, 90:10 Cu:N, and 70:30 Cu:N have been exposed to a number of microbial suites containing SRB. The following conclusions were drawn:

a. Under near-surface water conditions, formation of any copper sulfide mineral except possibly chalcocite ( $\text{Cu}_2\text{S}$ ) is indicative of corrosion induced by SRB. Data collected recently by another group indicates that chalcocite is a fingerprint, but data collected in this program do not constitute an independent demonstration.

b. Addition of nickel to copper alloys leads to formation of metastable djurelite ( $\text{Cu}_{1.9}\text{S}$ ) and hexagonal chalcocite ( $\text{Cu}_2\text{S}$ ) in corrosion products. The presence of major djurelite appears to correlate with adherent corrosion product films.

2. The so-called type 1 pitting corrosion of copper alloys, and the closely related "bronze disease" (which was the first form of corrosion conjectured to be microbiological) were analyzed in terms of a new type of stability diagram. This analysis indicates that type 1 pitting corrosion has an abiotic reaction path, but that its occurrence can be promoted by the presence of a biofilm, especially a biofilm containing acid-producing bacteria; parallel work on sulfating corrosion will be completed in FY 1991.

**SIGNIFICANCE:** The reason for studying mineralogical fingerprints is to discriminate microbiologically induced corrosion, in which the microbes responsible for the sulfides are in biofilms on the corroding surfaces, from microbiologically intermediated corrosion, in which microbes at a distance change the water chemistry. Only in the case of induced corrosion is the use of biocides appropriate. Identification of mineralogical fingerprints should be helpful in identifying SRB-induced corrosion of copper alloy systems.

**WORK PLAN (next 12 months):** The plan for Fiscal Year (FY) 1992 is to collect more data on the known mineralogical fingerprints of SRB-induced corrosion of iron and carbon steel. The research will address the following issues:

a. It is known that the lattice parameter and stoichiometry of synthetic mackinawite ( $\text{Fe}_{1.5}\text{S}$ ) produced abiotically in water at room temperature is different from that of natural mackinawite. No one has done a careful study of biogenic mackinawite to determine how its

lattice parameter relates to those of natural and abiotically synthesized samples. This will be the first priority in Fiscal Year (FY) 1992.

Research will be undertaken on the rates of formation and alteration of mackinawite and other biogenic iron sulfide minerals; electron diffraction and environmental scanning electron microscopy will be used. A better understanding of alteration kinetics will lead to an ability to judge from mineralogical observations the length of time SRB induced MIC has operated.

b. It is known that, in oxygenated seawater, mackinawite alters to "green rust 3," which is a complex iron hydroxysulfate. Experiments will be conducted to determine how rapidly oxygenated seawater alters the minerals produced on SRB-induced MIC of iron, and to determine whether the products of this alteration are themselves characteristic of SRB-induced corrosion.

In addition to the work on iron, some experiments will be conducted on copper alloys in order to facilitate the transfer of the copper MIC study to another research or exploratory development program.

PUBLICATIONS AND REPORTS (last 12 months):

Published or in press:

"Mackinawite Formation During Microbial Corrosion," by M.B. McNeil and Brenda J. Little, Corrosion 46, 599 (1990).

"Correlation of Laboratory Results with Observations on Very Long-Term Corrosion of Iron and Copper Alloys," by M.B. McNeil, David Mohr, and Brenda J. Little, in Proceedings of 1990 Materials Research Society Symposium in Materials Issues in Archeometry, Materials Research Society, Pittsburgh, PA (1991).

"Biocorrosion in Copper and Nickel Seawater Piping Systems," by B.J. Little, P.A. Wagner, Richard Ray, and M.B. McNeil, to appear in Marine Technology Society Journal 24, 10 (1990).

"A Geometric Study of Corroded Surfaces of Iron-Nickel Alloys," by D.W. Mohr and M.B. McNeil, Journal of Solid State Chemistry 88, 584 (1990).

"Microbiologically Influenced Corrosion of Silver in Marine Environments," by M.B. McNeil, to appear in Proceedings of the International Conference on Microbially Influenced Corrosion, National Association of Corrosion Engineers, Houston, TX (1991).

"Corrosion Products and Mechanisms in Long-Term Corrosion of Copper Alloys," by M.B. McNeil and B.J. Little, to appear in The Scientific Basis for Waste Management XIII, Materials Research Society, Pittsburgh, PA (1991).

"Mineralogical Fingerprints for Corrosion Processes Induced by Sulfate Reducing Bacteria," by M.B. McNeil, B.J. Little, and J.M. Jones, Corrosion 91 paper No. 580, National Association of Corrosion Engineers; Houston, TX (1991).

"Production of Sulfide Minerals by Sulfate Reducing Bacteria During Microbiologically Influenced Corrosion of Copper," by M.B. McNeil, J.M. Jones, and B.J. Little, to appear in Corrosion.



## ANNUAL PROGRESS REPORT

GRANT # N00014-89-J-1749

R&T CODE: 441h006

PRINCIPLE INVESTIGATOR: John Smit

INSTITUTION: University of British Columbia

GRANT TITLE: Chemical, structural and genetic analysis of the adhesive holdfast of biofouling *Caulobacters*.

REPORTING PERIOD: 28 June 1990 to 1 June 1991.

### OBJECTIVES:

- 1) Determine the chemical composition and structural arrangement of monosaccharides and other substituents within the holdfasts of selected marine and freshwater *Caulobacters*.
- 2) Further characterize the surfaces to which holdfasts will adhere.
- 3) Clone and analyze the genes specifying the holdfasts of selected marine and freshwater *Caulobacters*.
- 4) Continue developing the capabilities of selected marine *Caulobacters* for molecular genetic experimentation.
- 5) To evaluate the occurrence, stability and behavior of *Caulobacters* in complex biofilms, in collaboration with other ONR-funded researchers.

### ACCOMPLISHMENTS (past 11 months)

We are concentrating our efforts for in-depth holdfast composition and gene analysis on one marine *Caulobacter*, MCS6, and a freshwater *Caulobacter* (*Caulobacter crescentus* CB2A).

#### 1 Chemical analysis of the adhesive holdfast of *Caulobacters*

Our first efforts have been directed to CB2A. We have been concentrating on an isolation procedure based on the observation that holdfasts bind very tightly to colloidal gold particles. Once bound, the holdfast is no longer adhesive and the complex can be isolated readily by CsCl density centrifugation, relying on the high density imparted by the gold binding. We have isolated holdfast-"shedding" mutants that greatly assisted this process. We continue to have problems getting the holdfast sufficiently purified from other surface polysaccharides, yet our progress in analysis of these contaminants is allowing us to 1) select mutants that are missing offending polysaccharides and 2) identify monosaccharide peaks in gas chromatography that we know are not holdfast-derived and can ignore.

#### 3-On the genes that specify the holdfast structure

For the freshwater *Caulobacter crescentus* CB2A, we have learned that transposon Tn5-derived holdfast-defective mutants cluster in 4 regions throughout the genome and have a number of phenotypes. Among them was the "shedder" phenotype discussed above. During the past year we have been analyzing the genomic region of DNA for each of these groups and are presently narrowing down the regions expressing the relevant genes, by subcloning and complementation analysis. We have placed special emphasis of the shedder phenotype, because we believe the gene in question is responsible for anchoring the holdfast to the cell surface and therefore is the "perfect" substrate and therefore is a key approach to defining the molecular details of adhesion. We have defined 3 genes; 2 are transcriptional activators for a third gene, which we believe is the actual attachment protein. The transcriptional activators have a  $\phi$ 54-type

promoter, associated with developmental regulation in *Caulobacter*. We have cloned uninterrupted genome segments from the other 3 regions as well and are defining the minimum size of DNA that will complement the holdfast-defective phenotype produced by the Tn5 insertions, in preparation for DNA sequencing of these regions.

We repeated the gene identification and analysis process in the marine *Caulobacter* MCS6. We developed a library of 12,000 Tn5 insertion mutants and after developing a new screening assay for holdfast defective mutants (the one that worked well for the freshwater strains did not work for MCS6) we isolated 47 holdfast-defective mutants. Southern analysis has defined 3 regions of the genome that have holdfast related genes. Another large group (228 mutants) exhibit a holdfast that appears normal by visual techniques but is no longer adhesive. Preliminary Southern analysis indicates that a fairly large number of independent insertions are in this group; we are very interested in learning the exact defects.

4-Developing the capabilities of marine *Caulobacter* MCS6 for molecular genetic experimentation. During the reporting period we published a series of plasmid vectors, designed for gene expression in *Caulobacters*, including the marine MCS6. These will become important for future studies on holdfast gene regulation. During the reporting period we also published a procedure for introducing plasmids into marine *Caulobacters*. 5-The occurrence, stability and behavior of *Caulobacters* on surfaces and in complex biofilms, in collaboration with other ONR-funded researchers. David Stahl (U. of Illinois) and I concluded a study comparing the 16S rRNA sequences of marine and freshwater *Caulobacters*. One consequence was the identification of a nucleotide sequence that was apparently unique to the marine *Caulobacters* which can now be used to generate probes to directly assess abundance of marine *Caulobacters* in complex biofilms.

#### WORK PLAN:

The major shortterm goals are to 1) Finish the initial monosaccharide composition analysis of the CB2A holdfast and repeat the process for the marine *Caulobacter*, 2) Sequence and characterize the holdfast attachment gene of CB2A, 3) Characterize the genes within other holdfast-related genomic regions of CB2A and 4) Isolate and begin direct characterization of the holdfast-related genes of the marine *Caulobacter* MCS6.

#### PUBLICATIONS AND REPORTS RELEVANT TO THIS GRANT (last 11 months)

- MacRae, J. D., and J. Smit. 1991. Characterization of *Caulobacters* isolated from wastewater treatment systems. Appl. Environ. Microbiol. 57:751-58.
- Gilchrist, A., and J. Smit. 1991. Transformation of freshwater and marine *Caulobacters* by electroporation. J. Bacteriol. 173:921-925.
- Mitchell, D., and J. Smit. 1990. Identification of the genes affecting production of the adhesion organelle of *Caulobacter crescentus* CB2. J. Bacteriol. 172, 5425-5431.
- Bingle, W.H., and J. Smit. 1990. High level plasmid expression vectors for *Caulobacter crescentus* incorporating the transcription and transcription-translation initiation regions of the paracrystalline surface layer protein gene. Plasmid 24:143-148.
- Kurtz, H.D., and J. Smit. 1990. Preliminary characterization of the *Caulobacter* holdfast attachment protein. Annual Meeting, Northwest Branch, American Society for Microbiology. (Abstract).
- Ravenscroft, N., S.G. Walker, and J. Smit. 1990. The chemistry of adhesion in *Caulobacters*. International Conference: "Bioadhesion-A physico-chemical approach of biological adhesion in dentistry, medicine and industry", Groningen, Netherlands. (Abstract).

## Annual Progress Report

**GRANT #:** N00014-90-J-1932

**R&T CODE:** 441t005

**PRINCIPAL INVESTIGATOR:** Celia M. Smith

**INSTITUTION:** University of Hawaii

**GRANT TITLE:** Documentation of the Fouling Community in Pearl Harbor and Its Response to Anti-fouling Compounds

**REPORTING PERIOD:** 1 JUNE 1990 - 31 MAY 1991

**AWARD PERIOD:** 1 MAY 1990 - 30 SEPTEMBER 1991

**OBJECTIVE:** To test supplied coatings for anti-fouling capabilities in Pearl Harbor waters and to develop our understanding of the fouling community in that region.

**ACCOMPLISHMENTS:** We have begun an 18-mo. project to monitor the development of biological communities on non-toxic, anti-fouling coatings applied to standard surfaces. During Year 1, we duplicated many of the features of the Beaufort test program so that tests can be run in a parallel manner in the Pearl Harbor area.

Our test site was selected to challenge coatings with the "real world setting" of a naval base - heavy, near-year-round settlement and complex water qualities within Pearl Harbor. As of late fall, we were testing in the field and had rapid settlement of algae and invertebrates on coatings. We have expanded on this "real world challenge" by starting to develop a lab-based assay for fouling with larvae of the ubiquitous fouler in Pearl Harbor, *Hydroides elegans*.

Our approach for rapid assessment of settlement in the field is to employ a video-image analysis system. The components for this system have been purchased and are nearly debugged; we continue to work to improve image resolution for accurate measurements and counts of small organisms. We are now capable of automating routines for different kinds of analyses of images. One costly aspect of any field-based monitoring program is the labor intensive nature of characterizing settlement. Bringing a video image analysis system on line to facilitate and partly automate the acquisition and analysis of settlement data could become a breakthrough for this field.

Identification of settling organisms as well as their pattern for recruitment continues to gain insight into successional events. The general pattern of settlement has

the following stages: algae and hydroids by one week followed by appearance of *H. elegans* at about two weeks; bryozoans, sessile and colonial tunicates, polychaetes and barnacles appear at about four weeks whereby control rods are at  $\geq 100\%$  cover. Over the first months of these observations, we have seen the same sequence repeated but community development is more rapid in late spring - early summer than observed in January.

**SIGNIFICANCE:** The primary significance of testing in Pearl Harbor is the real life setting used to challenge the abilities of coatings to deter fouling. Already, we have observed Navy standard AF-121 foul with *H. elegans* in less than 3 weeks, about a week behind control rods (see above). Other significant potentials are our future contributions to basic principles of larval settlement and molecular aspects of surface composition that may help cue a larva or algal spore to settle.

**WORK PLAN:** Our goals for the remainder of this grant include: 1) identification of and relocation to a new site in Pearl Harbor (our current location at Yankee 3 will be under construction within 6 months); 2) final development of the settlement assay with *Hydroides elegans*; 3) compile marine plants and animals that foul treated and untreated rods and slides; 3) development of further statistical tests for quantitative evaluation of antifouling coatings; 4) gathering data to project rates of fouling as well as detail community structure and diversity needed to interpret fouling events - settlement, timing and spatial organization) and 5) the development and potential application of molecular probes to spore and larval surfaces for species identification and possible blocks to attachment to surfaces.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-88-K-0093

R&T CODE: 442s005

PRINCIPAL INVESTIGATOR: David A. Stahl

INSTITUTION: University of Illinois

GRANT TITLE: Characterization of Biofilm Microbial Community Structure  
by Ribosomal RNA Sequences

REPORTING PERIOD: 1 June 1990 - 1 June 1991

AWARD PERIOD: 1 July 1987 - 30 November 1991

OBJECTIVE: Develop molecular techniques for the identification and localization of microorganisms comprising natural biofilms. To use natural biofilm communities developed in bioreactors containing sulfate-reducing bacteria (SRB) and methanogens in order to: 1) validate techniques and 2) study the effects of changing reactor conditions (e.g. sulfate concentration) on diversity, activity and spatial relationships of biofilm populations. Longterm objectives include the identification and characterization of obligate symbioses with attached communities and studies of the relationship between community structure and activity.

### ACCOMPLISHMENTS:

Use of Fluorescent probes for measurement of single cell activity:  
During this project period we have initiated a study designed to evaluate the use of fluorescent probes for measurement of single cell activity within multispecies biofilms. The basis of the approach is the long observed correlation between growth rate and ribosome content. Since probe-conferred fluorescence is a measure of ribosome content, and ribosome content varies with growth rate, the fluorescence of individual cells could provide direct information of their in situ activity. To initiate this study we sought to isolate representative organisms of the bioreactor community. Pure culture studies would serve as a basis for direct observations of these populations within developing and established biofilms. Two such organisms were previously defined by our molecular characterizations. As detailed in the last report, group-specific PCR amplification and cloning were used to selectively retrieve 16S rRNA sequences from sulfidogenic populations. Two such sequences, one closely related to the sequence from Desulfovibrio vulgaris and the second to Desulfuromonas acetoxidans, were used to design specific oligonucleotide probes for these populations. The probes were initially used to microscopically visualize the corresponding organisms within multispecies biofilms (previous report).

During this project period, the probes were used to screen enrichment cultures. Selection of substrates and electron receptors (lactate or acetate and sulfate or sulfur, respectively) for enrichment was inferred from sequence relationships. An organism corresponding to the Desulfovibrio vulgaris-like bioreactor population (demonstrating specific hybridization) has been obtained in pure culture. Isolation of the Desulfomonas-like organism is ongoing.

The nutritional requirements of the Desulfovibrio vulgaris-like isolate will be characterized as a prelude to determining the relationship between growth rate and probe-conferred fluorescence. We are evaluating different approaches to quantitative fluorescence microscopy (e.g. photon counting imaging and confocal scanning microscope systems) in association with Dr. K.D. Wittrup, Chemistry, University of Illinois).

Environmental and Evolutionary Diversity of Caulobacter. The 16S rRNA

sequences of four caulobacters and hyphomonas isolates were determined (in addition to 13 sequences previously determined). Analysis of these sequences (in collaboration with ONR investigator John Smit, University of British Columbia) has shown the freshwater and marine varieties to be a diverse, yet phylogenetically coherent, assemblage. An early divergence within the main caulobacter lineage generally corresponds to freshwater and marine habitats. The sequence information is now serving for the design of group- and species-specific probes to identify and quantify these important biofilm organisms in the open environment (freshwater and marine) and bioreactor communities.

Global Nucleic Acid Hybridization Panel. An essential demonstration of any nucleic acid probe is target of group specificity. This demonstration for environmental studies is problematic since there is generally little knowledge of the overall population composition of natural microbial communities. We have approached the demonstration of rRNA probe specificity along two avenues. The first is the combined use of probes of identical or phylogenetically-nested specificity. Here, probe specificity is corroborated by the demonstration of comparable quantification of the environmental microbial target group by the combined use of the redundant or nested probes. The second avenue of probe verification is direct; the demonstration that nontarget organisms do not hybridize with the probe. To further develop this second approach to verification, we have assembled an extensive collection of nucleic acids from a spectrum of organisms spanning most of the now recognized phylogenetic diversity (65 organisms). This diversity of nucleic acids, immobilized to a single nylon membrane ("global membrane"), will serve as a reference panel for the routine characterization of hybridization probes.

SIGNIFICANCE: The definition of microbial populations is essential for studies of the community ecology (succession, diversity, population associations and activities). Control and manipulation of biofilms should benefit from understanding of community architecture and the activity of individual contributing populations. For example, our previously reported observation that sulfate-reducing bacteria are apparently abundant both in the presence and absence of sulfate should be considered in approaching environmental control.

WORK PLAN: Continued research will emphasize the use of perturbation/response studies (varying amount, or species of, electron acceptors, addition of inhibitors or fermentation intermediates). Single cell activity, and temporal and spatial relationships of methanogenic and sulfidogenic biofilm populations, will be evaluated using developing and established molecular criteria. Specific associations (symbioses) suggested by concerted population shifts (as inferred by DNA probing) will be microscopically characterized using the corresponding fluorescent probes. Under steady-state reactor conditions, the order and character of microbial colonization will be examined. We will continue to evaluate the use of mathematical models for predicting the function and community composition of these bioreactor systems.

#### PUBLICATIONS AND REPORTS:

##### **Book chapter**

Stahl, D.A., and Amann, R. 1991. Development and application of nucleic acid probes in bacterial systematics. In: Sequencing and Hybridization Techniques in Bacterial Systematics. pp. 205-248. E. Stackebrandt and M. Goodfellow (Eds.), John Wiley and Sons, Chichester, England.

ANNUAL PROGRESS REPORT

GRANT #: R&T CODE: 441z013

PRINCIPAL INVESTIGATOR: David A. Stahl

INSTITUTION: University of Illinois

GRANT TITLE: Identity, Spatial Distribution and Activity of  
Microbial Populations in Natural Biofilms

REPORTING PERIOD: 1 April 1991 - 1 July 1991

AWARD PERIOD: 1 April 1991 - 31 March 1993

OBJECTIVE: To use molecular probes to elucidate key biochemical transformation processes that govern anaerobic mineralization through consortial interactions of sulfate-reducing and methanogenic bacteria which structure biofilm communities.

ACCOMPLISHMENTS: This is a new grant.

GRANT #: N00014-90-J-1410

R&T CODE: 441h008

PRINCIPAL INVESTIGATOR: T.R. Tosteson (PI) and Y. Yamamura

INSTITUTE: University of Puerto Rico, Mayaguez Campus

GRANT TITLE: The Molecular Specificity of Biofilm Macromolecules  
in Microbial Biofouling of Artificial Surfaces in the Sea

REPORTING PERIOD: 1 July, 1990 - 1 June, 1991

AWARD PERIOD: 1 February, 1990 - March 31, 1992

OBJECTIVE: To assess variability and diversity of microbial aggregation and adhesion enhancing macromolecules (AAEM) found in solution in ambient seawater and on biofouled surfaces employing immunological techniques. Monoclonal antibodies (MAbs) raised against AAE macromolecules will be used to chromatographically isolate and purify individual classes of AAE macromolecules from mixtures of such components. The specificity of the interactions of the AAEM with glass, metallic and cell surfaces will be assessed.

ACCOMPLISHMENTS (last 12 months): AAEM have been isolated from particulate free coastal seawater and microalgal culture media by hydroxylapatite (HAP) chromatography. The components recovered from each of these sources were fractionated by ultrafiltration (Amicon) into three size classes, > 100k, 100k to 30k and 30k to 10k daltons. While all size fractions showed some AAE activity, the material in the 100k to 30k size range was the most active, significantly enhancing the adhesion of the test micro-algal cells at pg/ml concentrations. Groups of mice (5 to 6 animals each) were hyperimmunized with newly isolated AAEM as well as AAEM antigens recovered in older preparations. The hyperimmune sera in both of these cases significantly enhanced (6 to 10X) the adhesion of the test cells. This effect was further increased (2X) in the presence of antigen at  $10^{-15}$  g/ml, and inhibited at higher concentrations of AAEM antigen.

SIGNIFICANCE: The micro-algal adhesion assay detects the presence of anti-AAEM antibodies in the sera of hyperimmunized mice. The cell surface bound AAEM will provide an important tool for the isolation of AAEM antibodies from the sera of hyperimmunized mice and more importantly, provide a means of screening hybridomas for the production of immunoglobins reactive with hyperimmune, anti-AAEM mouse sera. Identification of the specific AAEM components that interact with their complimentary elements on microbial cell surfaces will provide an important tool for the analyses of the interaction of macromolecular surface films and microbial cell surfaces in biofouling.

WORK PLAN: (next 12 months): The immediate objectives of the coming year are; (1) to complete the screening of hybridomas made to the new size fractionated AAEM and older antigens using the adhesion assay and (2) establish which of the positive hybridoms produce MAbs that selectively bind individual AAEM. Following this, the diversity and nature of the interactions of AAEM with glass plastic, metallic and cell surfaces will be examined.



PUBLICATIONS AND REPORTS (last 12 months):

1. Publications:

Tosteson, T.R., R. A. Edwards and D.G. Baden. 1990. Polyether Dinoflagellate Toxins and Caribbean Ciguatera: Correlation of Fish Toxins with Standard Toxins. Proceedings of the Third International Conference on Ciguatera Fish Poisoning. La Parguera, Puerto Rico, in press.

Escalona de Motta, G., J.A. Mercado, T.R. Tosteson and D.L. Ballantine. 1990. Inhibition of Skeletal Muscle Response to Acetylcholine by Dinoflagellate and Ciguatoxic Fish Extracts. Proceedings of the Third International Conference on Ciguatera Fish Poisoning. La Parguera, Puerto Rico, in press.

**BIOLUMINESCENCE**

**CORE PROGRAM**

**SCIENTIFIC OFFICER: DR. RANDALL S. ALBERTE**

**PROGRAM OBJECTIVE: TO DETERMINE HOW LIGHT PRODUCTION IN THE MARINE ENVIRONMENT IS REGULATED.**

**NAVY OBJECTIVE: TO PROVIDE A FRAMEWORK FOR ASSESSING AND PREDICTING THE LIGHT CONTRIBUTION FROM BACTERIAL LUMINESCENT SOURCES IN THE OCEAN.**

# ANNUAL PROGRESS REPORT

GRANT #: N00019-88-K-0570

R&T CODE: 441d019

PRINCIPAL INVESTIGATOR: Everett P. Greenberg

INSTITUTION: University of Iowa

GRANT TITLE: Regulation of *lux* Genes in *Vibrio fischeri*: Control of a Symbiosis-related Gene Expression System in a Marine Bacterium

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 June 1990 - 31 May 1991

OBJECTIVE: To investigate the mechanism of autoinduction of the *Vibrio fischeri lux* genes and to understand some of the physical factors that affect autoinduction. Specific objectives include developing an understanding of the nature of the autoinducer interaction with the receptor, LuxR, and the nature of the LuxR interaction with *lux* DNA.

ACCOMPLISHMENTS (last 12 months): We have completed our 5' deletion analysis of the *luxR*. Our previous point mutational analysis indicated that amino acid residues 79-127 of the 250-residue LuxR protein form an autoinducer-binding region, and residues 184-230 include the DNA-binding region. The deletion analysis demonstrates that as is true of the wild-type LuxR, truncated LuxR proteins with N-terminal deletions through the autoinducer-binding region are capable of activating luminescence gene transcription. Unlike the wild-type protein, the N-terminal deletion proteins are autoinducer independent. In fact, proteins containing the C-terminal 30% of LuxR appear to be fully active as transcriptional activators. These data support a model whereby the N-terminal region of LuxR serves to mask the activity of an independently folded C-terminal domain that is the *lux* gene activator. According to this model, autoinducer binds to the N-terminal domain and alters it in such a way as to unmask the activity of the C-terminal domain. In related experiments, it was demonstrated that the N-terminal region of LuxR is required for a second function. In addition to masking the activity of the C-terminal domain, the N-terminal region is necessary for negative autoregulation of *luxR* transcription.

Attempts to study the activity of purified LuxR in vitro have stalled while we test the hypothesis that this protein is membrane bound. If indeed the suggestion that LuxR is membrane bound proves to be correct, it would have a major influence on our approach to in vitro studies. Although *luxR* does not code for an N-terminal signal peptide, the LuxR protein does contain two potential membrane spanning hydrophobic stretches. Therefore we have applied TnphoA fusion technology in hopes of obtaining information about the cellular location of LuxR. To date we have one TnphoA that in fact directs *E. coli* to synthesize active alkaline phosphatase and we have demonstrated that this fusion protein fractionates with membranes. The locations of this fusion (alkaline phosphatase is fused to LuxR residue-139) is in a region that should reside on the periplasmic side of LuxR assuming a hypothetical model based on the two potential membrane spanning regions. One must be cautious with interpretations based on a single fusion. We are currently generating ten additional fusions by site-directed mutagenesis.

We have also continued to study *V. fischeri* ES114, a specific symbiont from the light organ of the squid, *Euprymna scolopes*. This strain does not produce light in the laboratory unless autoinducer is added to the medium, and we are interested in why it does not produce autoinducer. We have cloned the ES114 luminescence gene cluster and we

have demonstrated that 1) the genes are organized in a fashion similar to those in autoinducer synthesizing strains, 2) the *luxI* gene directs *E. coli* to synthesize autoinducer, and 3) even when *luxI* transcription is activated in ES114, this strain makes very little autoinducer in laboratory culture. Thus ES114 is either incapable of producing a substrate for autoinducer synthase (which could be provided by the squid), there is posttranscriptional control such that ES114 contains little or no synthase, or there is an inhibitor of synthase activity in ES114 grown under standard laboratory conditions.

**SIGNIFICANCE:** The 5'-deletion analysis supports a structural model of LuxR in which there are two modules. A C-terminal transcriptional activator module, and an N-terminal module that can mask the activity of the C-terminal module. Apparently the binding of autoinducer to the N-terminal module inhibits the masking activity. The N-terminal model is also required for negative autoregulation of *luxR*. The module concept has implications regarding the evolution of LuxR and other members of the LuxR family (also referred to as the FixJ or the UhpA family). The identification of an independently folded C-terminal domain suggests a number of other experiments that should yield a greater understanding of the structure and function of LuxR. The application of *TnphoA* fusion technology will now allow us to establish the cellular location of LuxR. At this time, it appears that LuxR may be compartmentalized to the cytoplasmic membrane. Finally the studies of the squid symbiont allow us to begin to study the development of the symbiosis at the molecular level.

**WORK PLAN (next 12 months):** We plan to complete the *TnphoA* mutagenesis studies. We will also embark on a 3' deletion analysis of *luxR* that should complement our 5' deletion analysis. We will determine the specific defect in ES114 leading to the inability of this strain to synthesize significant quantities of autoinducer, and we will initiate experiments to determine the significance of the *lux* genes to infection of squids by ES114.

**PUBLICATIONS AND REPORTS (last 12 months):**

1. Slock, J. D. VanRiet, D. Kolibachuk, and E. P. Greenberg. 1990. Critical regions of the *Vibrio fischeri* LuxR protein defined by mutational analysis. *J. Bacteriol.* 172:3974-3979.
2. Dunlap, P. V., and E. P. Greenberg. 1991. The role of intercellular chemical communication in the *Vibrio fischeri*-Monocentrid fish symbiosis. (In press).
3. Choi, S.H., and E.P. Greenberg. 1991. The C-terminal region of the *Vibrio fischeri* LuxR protein contains an inducer-independent *lux* gene activating domain. Submitted for publication.
4. Choi, S.H., and E.P. Greenberg. 1991. Activation of the *Vibrio fischeri* luminescence genes by truncated LuxR proteins is autoinducer independent. Abstr. Annual Meet. Amer. Soc. Microbiol. 91.
5. Gray, K.M., and E.P. Greenberg. 1991. Cloning and characterization of the luminescence gene cluster from *Vibrio fischeri* ES114, the light organ symbiont of the sepiolid squid *Euprymna scolopes*. Abstr. Annual Meet. Amer. Soc. Microbiol. 91.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-88-K-0130

R&T CODE: 4412033

PRINCIPAL INVESTIGATOR: J. Woodland Hastings

INSTITUTION: Harvard University, Department of Cellular and Developmental Biology

TITLE: Molecular Biology and Genetic Regulation in Marine Dinoflagellates

REPORT PERIOD: 31 May 1990 - 27 March 1991

AWARD PERIOD: 1 June 1988 - 31 May 1991

OBJECTIVE: To investigate the structure and organization of the genome of marine dinoflagellates, as well as the mode whereby its expression is controlled.

### ACCOMPLISHMENTS:

#### 1) Luciferin Binding Protein (LBP) cDNA Sequencing:

During the first year of this three year project, a partial (~1 kb) cDNA for LBP, corresponding to the 3' end of the mRNA was partially sequenced. This clone was used for analyzing the distribution of LBP mRNA via Northern blot analysis. In the next year, we completed the sequencing of this clone. A potential reading frame for the LBP mRNA was deduced on the basis of the occurrence of only a single stop codon, with a 3' untranslated region of 139 bases. No obvious signal sequence for polyadenylation was detected, based on the known consensus sequences for polyadenylation in eukaryotes.

#### 2) Full length LBP cDNA and structure of LBP mRNA:

Primer extension analysis, using a primer located near the 5' region of other existing cDNA, shows a region of strong secondary structure immediately upstream of the known end of the first cDNA clone (approx. 1000 nt long). This is indicated by the occurrence of premature stops of the reverse transcriptase (AMV RT, 45°C) at several points in the mRNA. Indeed, severe denaturing conditions (~100°C, MeHgOH) are required to unfold the mRNA for any primer extension to take place past this point, and even with this treatment, only 5-10% extension occurs past this region. A  $\lambda$  gt10 sub-library, primed with an LBP-specific oligonucleotide, was screened to recover the upstream sequence. Two additional clones were recovered, but both terminated at the initial region of high secondary structure. This occurred even though extensive denaturation of the mRNA was performed to eliminate secondary structure. A second lambda sub-library, transcribed with an improved reverse transcriptase<sup>RT</sup> and excess template (~100  $\mu$ g RNA) gave 15 additional clones, one of which appears to represent a full-length clone. This clone has been >90% sequenced, and an open reading frame established, coding for a protein of ~630 amino acids.

#### 3) Cloning of the LBP gene:

Through Southern blotting analysis of the restricted DNA with LBP cDNA probes, DNA fragments which gave rise to positive hybridization signals were isolated and utilized as templates for the inverse polymerase chain reaction (I-PCR, ref. Ochman et al. *Genetics* 1988, 120: 621). The amplified DNA products were cloned into plasmid vectors. Complete nucleotide sequences of these cloned LBP gene fragments have been determined, and selected fragments subcloned for further sequence determinations. Currently, an additional sequence of ~300 nt upstream from the transcription start has been analyzed. This region probably contains the *Gonyaulax* promoter. No sequence differences were found

between the genomic and cDNAs in the regions examined, indicating that no introns occur within these sequences.

**4) Gene copy number and/or gene families:**

To determine the organization of the LBP gene (or genes) in the genome, the LBP cDNA and its sequence information were utilized.

a) Southern blot analysis of restricted and unrestricted genomic DNA with the LBP cDNA probe suggests that there are multiple copies (and/or gene families) present. Indeed, there appear to be at least 100 copies of the gene (or pseudogenes) present.

b) The possibility of the presence of either gene families and/or pseudogenes of LBP was further studied by PCR analysis, in which the amplified product was produced from total genomic DNA using a set of oligonucleotide primers for the LBP gene. While the interpretation of these data is still underway, it appears that there is only one LBP sequence in the *Gonyaulax* genome.

c) Pulsed field electrophoresis (PFE) of *Gonyaulax* chromosomes indicates that the dinoflagellate chromosome is approximately 3.5 megabases in size, with most chromosomes of approximately equal size. This is comparable to yeast chromosome sizes.

**SIGNIFICANCE:** The dinoflagellates are one of the most important members of the phytoplankton in the marine ecosystem (and in fresh water as well). In terms of productivity, they lie at the base of food chains and carry out the photosynthetic energy capture upon which all other organisms are dependent. They are also responsible for massive blooms (red tides), sometimes involving fish kill. Some members produce highly potent neurotoxins, and some are responsible for the brilliant bioluminescence ("phosphorescence") of the ocean. This is seen at night when the water is disturbed, in the wake of a ship or submarine. A basic understanding of genetic regulation of these systems will enhance our insight into such phenomena.

Phylogenetically the dinoflagellates remain something of an enigma, partly because of the several unusual features of the nucleus. These include the permanently condensed chromosomes, which lack histones and also possess an unusually high DNA content (2-60 times that of the human nucleus!), with a high amount of a modified base. An understanding of the functional significance of these and other features of the nucleus could contribute to our understanding of many aspects of the ecology and dynamics of the ocean.

**WORK PLAN (next 12 months):** We are presently analyzing the 5' untranslated region for target sequences which are responsible for translational control, by gel shift assays and by construction of chimeric transcripts. We are also transfecting genomic LBP sequences into *Chlamydomonas* to identify regulatory elements.

**PUBLICATIONS AND REPORTS:** none

ANNUAL PROGRESS REPORT

GRANT #: R&T CODE: 441z002

PRINCIPAL INVESTIGATOR: Dr. Anthony P. Mahowald

INSTITUTION: The University of Chicago

GRANT TITLE: Regulation of Light Output and Identification  
of Luminous Bacteria from Free-Living and  
Particle-Associated Bacterial Assemblage

REPORTING PERIOD: 15 February 1991 - 1 July 1991

AWARD PERIOD: 15 February 1991 - 14 February 1994

OBJECTIVE: Determine natural abundances and genetic  
variation in light out-put properties of luminous bacteria  
and ascertain the primary environmental factors that control  
lux gene expression and light generation.

ACCOMPLISHMENTS: This is a new grant.

ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 441z008

PRINCIPAL INVESTIGATOR: Kenneth H. Nealson

INSTITUTION: University of Wisconsin

GRANT TITLE: Distribution and Activities of Bioluminescent  
Bacteria: Physiological and Molecular  
Approaches

REPORTING PERIOD: 15 February 1991 - 1 July 1991

AWARD PERIOD: 15 February 1991 - 31 December 1991

OBJECTIVE: Determine the role of temperature, iron availability and salinity in controlling the capacity for light production by two major groups of bioluminescent bacteria and then develop probes that can be used to assess the impact of these environmental factors on physiological performance of bacteria.

ACCOMPLISHMENTS: This is a new grant.



ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 441z010

PRINCIPAL INVESTIGATOR: Dr. Dennis J. O'Kane

INSTITUTION: University of Georgia Research Foundation,  
Inc.

GRANT TITLE: Factors Determining the Natural and Genetic  
Diversity of Marine Bacteria with *Vibrio*  
*harveyi*-type.

REPORTING PERIOD: 1 March 1991 - 1 July 1991

AWARD PERIOD: 1 March 1991 - 28 February 1994

OBJECTIVE: To determine the primary environmental factors  
characteristic of coastal and estuarine environments that  
control the growth and abundance of *Vibrio harveyi* and  
determine the role of horizontal gene transfer in genetic  
differentiation of the species.

ACCOMPLISHMENTS: This is a new grant.

ANNUAL PROGRESS REPORT

GRANT #: R&T CODE: 441z012

PRINCIPAL INVESTIGATOR: Edward G. Ruby

INSTITUTION: University of Southern California

GRANT TITLE: The Role of Light Organ Symbiosis in the  
Distribution and Diversity of the Marine  
Luminous Bacterium *Vibrio fischeri*

REPORTING PERIOD: 15 February 1991 - 1 July 1991

AWARD PERIOD: 15 February 1991 - 14 February 1994

OBJECTIVE: Determine the role of dormancy as a factor  
controlling light generation by luminous bacteria in the  
ocean and use this information to develop probes to assess  
this state. In addition, the distribution of potentially  
symbiotic luminous bacteria will be determined in natural  
temperate and tropical waters.

ACCOMPLISHMENTS: This is a new grant.

ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 441z009

PRINCIPAL INVESTIGATOR: Michael R. Silverman

INSTITUTION: The Agouron Institute

GRANT TITLE: Role of Bioluminescence in Survival of  
Bacteria in the Marine Environment

REPORTING PERIOD: 15 February 1991 - 1 July 1991

AWARD PERIOD: 15 February 1991 - 23 July 1992

OBJECTIVE: Define the environmental controls on bacterial  
bioluminescence in relation to the molecular genetic  
mechanisms that govern gene expression and light output.

ACCOMPLISHMENTS: This is a new grant.

## ANNUAL PROGRESS REPORT

GRANT#: N00014-90-J-4065 R&T CODE: 41h002

PRINCIPAL INVESTIGATOR: Michael R. Silverman

INSTITUTION: The Agouron Institute  
505 Coast Boulevard South  
La Jolla, CA 92037

GRANT TITLE: Role of Bioluminescence in Survival of  
Bacteria in the Marine Environment

REPORTING PERIOD: 24 July 1990 - 23 July 1991 (12 months)

AWARD PERIOD: 24 July 1990 - 23 July 1992

OBJECTIVE: To investigate the function of bioluminescence of *Vibrio harveyi*. Specifically, to construct mutants with defined mutations in bioluminescence genes, *lux*, and to examine the growth and viability of mutants in a variety of stress-inducing physiological conditions.

ACCOMPLISHMENTS (last 10 months): We have been constructing Lux<sup>-</sup> mutants of *Vibrio harveyi* by a gene replacement method. This involves inactivating cloned *lux* genes in recombinant *E. coli* by transposon Tn5 insertion, mobilizing the mutated genes into *V. harveyi* by conjugation on a broad-host-range cloning vector, and then isolating recombinants of *V. harveyi* containing the mutant *lux* allele by selecting for inheritance of the selectable marker on the Tn5 transposon. Mutants with defects in the genes encoding the enzymes for bioluminescence (*luxC,D,A,B,E*) and in the regulatory gene (*luxR*) have been constructed.

Bioluminescence could function as an alternative pathway in energy metabolism, so its role in the cell might be revealed if other pathways of electron flow were eliminated. Specifically, double mutants with defects in luminescence and respiration were sought. Strains with conditional respiration defects were isolated by screening a library of transposon miniM<sub>lac</sub>-induced insertion mutants for those with iron uptake defects. Initial characterization of these mutants indicated that a siderophore-mediated iron uptake system had been inactivated, and in the absence of an appropriate iron source these mutants were respiration deficient due to the inability to synthesize cytochromes. The set of *lux* gene mutations was then recombined into these iron uptake mutants to produce a collection of double mutants.

Growth of Lux<sup>-</sup> mutants in a balance minimal sea water medium was little different from that of the wild type. However, double mutants had a significantly longer generation time as compared to that of the mutants with a single *lux* defect or a defect in the iron uptake system. So, bioluminescence appears to be important to the growth of *V. harveyi*, but dependence on this function was apparent only when respiratory electron flow was interrupted.

SIGNIFICANCE: Observation of a growth defect resulting from a *lux* mutation suggests that bioluminescence can, under some circumstances, be of benefit to the bacterium, and, given the capability for refined genetic

manipulation of *V. harveyi*. it should be possible to develop a more precise understanding of the function of bioluminescence.

**WORK PLAN (next 14 months):** Additional *lux* genes involved in regulation of bioluminescence are currently being characterized in another project in the laboratory, and mutants with defects in these genes will be integrated into this study. Regulatory genes could be members of global networks controlling many gene systems, and analysis of their function could reveal connections between bioluminescence and other cellular functions. And, more mutants which contain *lux* mutations in combination with other defects, such as those in the iron-uptake system, will be constructed. Double mutants could be particularly important because luminescence is a dispensable function which appears to be significant only when other functions are missing or possibly when unusual circumstances are encountered. The regimen of environments to which the *Lux*<sup>-</sup> mutants have been exposed has been limited, and the growth and long-term viability of mutants will be evaluated in more media, specifically nutrient-limited ones which induce particular starvation or stress responses.

**PUBLICATIONS:**

1. M. Silverman, R. Showalter and L. McCarter (1991) Genetic Analysis in *Vibrio*. *Methods in Enzymology* 204:in press.

ANNUAL PROGRESS REPORT

GRANT #: R&T CODE: 441z004

PRINCIPAL INVESTIGATOR: Keith B. Ward

INSTITUTION: Naval Research Laboratory

GRANT TITLE: Structure/Function Studies of Marine  
Bioluminescence Systems using Protein  
Crystallography

REPORTING PERIOD: 15 February 1991 - 1 July 1991

AWARD PERIOD: 15 February 1991 - 15 February 1994

OBJECTIVE: Single crystal x-ray diffraction analyses on marine bioluminescent proteins will be performed in order to elucidate the structure-function relationships that influence the bioluminescent signal intensity and frequency. This work will be performed on gene products of both native and site-directed mutant proteins that will be provided by collaborators at Texas A&M University. Initial work will focus on the *V. harveyi* but will be extended to include luciferases from other bacteria as well as the important accessory proteins, yellow fluorescent protein and lumazine-binding protein.

ACCOMPLISHMENTS: This is a new grant.

ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 441z003

PRINCIPAL INVESTIGATOR: Dr. David C. White

INSTITUTION: The University of Tennessee

GRANT TITLE: Monitoring Microfouling Ecology with  
Bioluminescent Bacteria

REPORTING PERIOD: 1 February 1991 - 1 July 1991

AWARD PERIOD: 1 February 1991 - 1 February 1994

OBJECTIVE: To determine the effects of consortial interactions and other physical or chemical parameters on the adhesion, invasiveness, metabolic activity, bioluminescence, and succession/persistence of bioluminescent bacteria in marine biofilms.

ACCOMPLISHMENTS: This is a new grant.

ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 441z001

PRINCIPAL INVESTIGATOR: Miriam M. Ziegler

INSTITUTION: Texas A&M Research Foundation

GRANT TITLE: The Three Dimensional Structure of Bacterial  
Luciferase

REPORTING PERIOD: 15 February 1991 - 1 July 1991

AWARD PERIOD: 15 February 1991 - 14 February 1994

OBJECTIVE: To obtain high quality crystals of bacterial luciferase in order to determine the three-dimensional structure to less than 3 Å and to design recombinant proteins with altered light generation and spectral properties.

ACCOMPLISHMENTS: This is a new grant.



**BIOMIMETIC MOLECULES AND PROCESSES**

**ARI (JOINT WITH CHEMISTRY DIVISION)**

**SCIENTIFIC OFFICER: DR. HAROLD J. BRIGHT**

**PROGRAM OBJECTIVE: TO PROCEED FROM THE PRINCIPLES OF MOLECULAR RECOGNITION AND ENZYME MECHANISM TO THE DESIGN AND SYNTHESIS OF CATALYTIC SITES AND SIMPLIFIED (DIFFUSIBLE) GROUP TRANSFER COENZYMES.**

**NAVY OBJECTIVE: TO DEVELOP THE BASIC SCIENCE REQUIRED TO DESIGN AND SYNTHESIZE CATALYSTS AND RECEPTORS CAPABLE OF DIRECTING THE CONSTRUCTION OF NON-PETROLEUM BASED MATERIALS AND OF DETECTING AQUEOUS ANALYTES OF INTEREST TO THE NAVY.**

# ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1697

R&T CODE: 441s007

PRINCIPAL INVESTIGATOR: Stephen J. Benkovic

INSTITUTION: The Pennsylvania State University

GRANT TITLE: Catalytic Antibodies

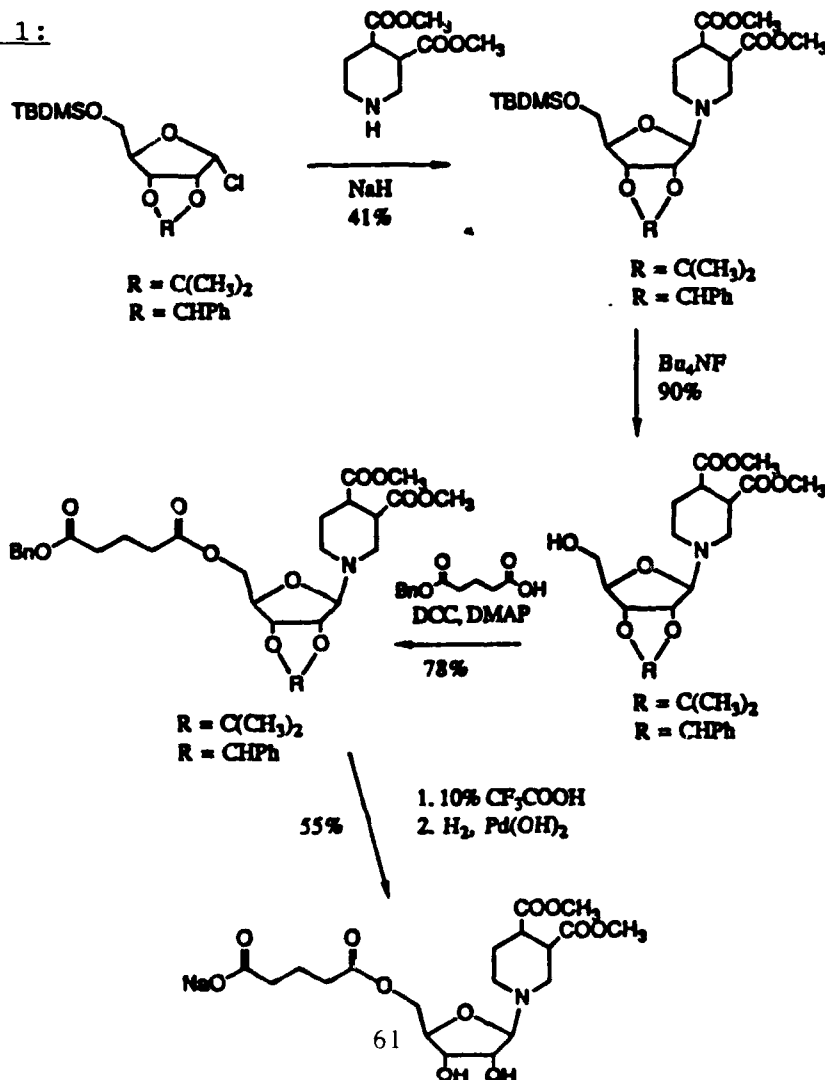
REPORTING PERIOD: 15 March 1990 - 14 March 1991

AWARD PERIOD: 15 March 1990 - 14 March 1993

**OBJECTIVE:** To induce a catalytic antibody that utilizes a nicotinamide base cofactor as a source of oxidizing equivalents.

**ACCOMPLISHMENTS (last 12 months):** Synthesis of a 3,4-disubstitute pyridine followed by its reduction in the presence of platinum oxide furnished the tetrahydropyridine needed for the desired hapten. Coupling of the tetrahydropyridine to a halogenose followed by stepwise deprotection then conjunction to the linker furnished the desired hapten as outlined in Scheme 1.

Scheme 1:



We were able to compare the end products by  $^1\text{H}$  NMR and  $^{13}\text{C}$ -NMR and showed that both are a mixture of the four expected stereoisomers with an  $\alpha:\beta$  ratio of about 1:4.

The hapten recently has been injected into mice and some twenty monoclonal antibodies have been prepared. We are presently testing them for their ability to catalyze the oxidation of formic acids as well as investigating the redox potential of the corresponding ribosyl nicotinamide in the binding pocket of the antibody.

SIGNIFICANCE: The project represents the first exploration of use of catalytic antibodies in redox reactions featuring nicotinamide cofactors.

WORK PLAN (next 12 months): Objectives for the next research period is of course to test the twenty monoclonal antibodies for their ability to catalyze the oxidation of formic acid. However, in addition we will investigate the binding to the antibodies of the oxidized and reduced forms of ribosyl niconinamide and determine whether the redox potential is markedly altered by the presence of the cofactor in the antibody binding pocket. In addition, we will investigate whether the antibody catalyzes the formation of 1,4-dihydroadducts such as the addition of cyanide to the oxidized form of the cofactor. Our efforts will be directed at surveying the twenty or so antibodies for catalytic properties and choosing then one to examine in some detail.

PUBLICATIONS AND REPORTS (last 12 months): The synthetic portion of this work was presented as a poster at the 26th Burgenstock Conference in Luzerne, Switzerland, April 28 - May 4, 1991.

# ANNUAL PROGRESS REPORT

Grant #: N00014-90-J-4132

R&T CODE: 441n048

PRINCIPAL INVESTIGATOR: Thomas C. Bruice

INSTITUTION: University of California at Santa Barbara

GRANT TITLE: The Dual and Simultaneous Roles of Nucleophilic Delivery and Assistance of Leaving Group Departure by Metal Ions in Phosphate Transfer From Phosphate Diesters.

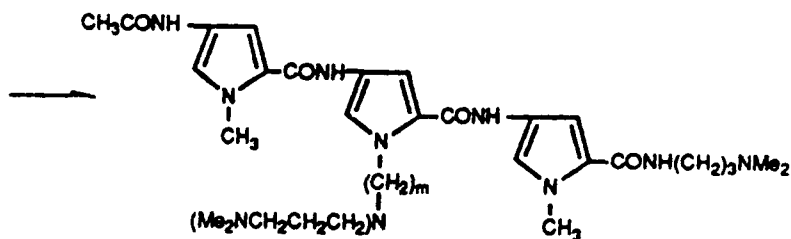
REPORTING PERIOD: 14 September 1990 - 20 May 1991

AWARD PERIOD: 14 September 1990 - 13 September 1992

OBJECTIVE: We propose that the facile hydrolysis of phosphate diesters can be achieved with synthetic catalysts containing two metal ions. One such catalyst center would involve: (i) complexation of both metal ions to pro-R and pro-S phosphate oxygens; (ii) one of the metal centers would hold HO<sup>-</sup> in proper position for in-line nucleophilic displacement; and (iii) the second metal center would be in position to complex the incipient alcoholate leaving group.

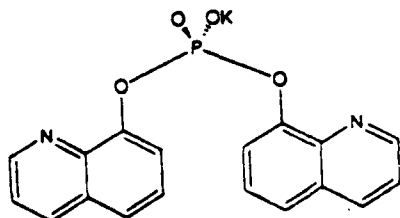
## ACCOMPLISHMENTS:

SYNTHETIC RESTRICTION ENZYMES. Studies of the x-ray structures of distamycin bound in the minor groove of d(C-G-C-A-A-A-T-T-T-G-C-G)<sub>2</sub> show that the central pyrrole nitrogen is located just behind a phosphodiester linkage. Our approach to *synthetic restriction enzymes* has been to replace the N-methyl substituent on the central pyrrole of distamycin by side chains terminating in metal complexing groups. We have completed the synthesis of compounds I and II. Experiments with DNA binding have been initiated. We will then determine if I and/or II are capable of catalyzing the cleavage DNA.



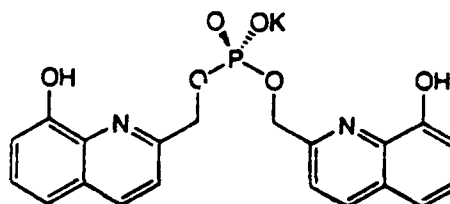
m=3, Compound I  
m=4, Compound II

INTRAMOLECULAR MODELS. The observed rate constant for phospho-diester III is given by equation 1.



Compound III

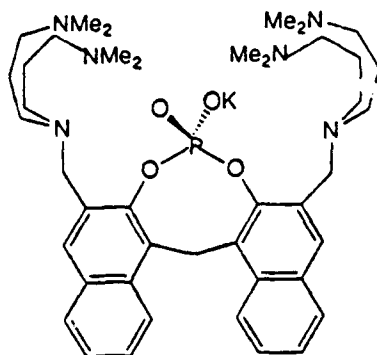
$$k_{\text{obsd}} = k_1 K_{a1} / (K_{a1} K_{a2} + K_{a1} a_H + a_H^2)$$



Compound IV

We suggest that the reactive ester species is monoprotinated at a quinoline nitrogen. It is possible that the hydrolysis results from concerted general-acid catalysis by protonated quinoline nitrogen and general-base catalysis by unprotonated quinoline nitrogen. Studies of metal ion catalysis of the hydrolysis of III have been initiated.

The synthesis of the phosphodiester IV is in process. Compound V has been a challenge.



Compound V

**SIGNIFICANCE:**

The synthesis of compounds I and II mark a new approach in the design of minor groove DNA binders and nuclease mimics. We use distamycin as a truck to carry putative catalytic centers. If I and II are not catalysts, we will find other analogues which will be.

**WORK PLAN:** To continue in the directions indicated in the initial request.

**PUBLICATIONS AND REPORTS:** None

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-4089

R & T CODE: 441s011 01

PRINCIPAL INVESTIGATOR: Donald Hilvert

INSTITUTION: Research Institute of Scripps Clinic

GRANT TITLE: Genetic selection for improved abzymes in *E. coli*.

REPORTING PERIOD: 1 September 1990 - 31 August 1991

AWARD PERIOD: 1 September 1990 - 31 August 1993

OBJECTIVE: To exploit molecular biological techniques and classical genetic selection to improve the chemical efficiency of first-generation antibody catalysts.

ACCOMPLISHMENTS: We are initially studying the abzyme 1F7 to evaluate the feasibility of employing *E. coli* as a host for the proposed genetic experiments. This antibody possesses chorismate mutase activity and the genes encoding it were previously cloned and sequenced in our lab and successfully expressed as functional Fab protein in yeast. To date we have investigated two approaches for expressing 1F7 in *E. coli*: 1) the lambda phage system developed in the Lerner lab at Scripps, and 2) the Genex single chain antibody approach.

We inserted the cloned heavy and light chain gene fragments of 1F7 into the Lerner bacteriophage vectors and transfected *E. coli* with the resulting constructs. Although ELISA measurements indicated that both heavy and light chains were biosynthesized upon induction, the amount of functional Fab (i.e. antigen-binding protein) was very low and we were never able to isolate enough Fab protein for detection by gel electrophoresis, much less for kinetic analysis. The Lerner lab has also reported low yields for individual antibodies, but 1F7 may be particularly problematic (vide infra). We encountered similar difficulties in preparing combinatorial Fab libraries from a mouse that had been hyperimmunized with our hexachloronorbornene "Diels-Alderase" hapten. Even though the experiments were carried out with the help of one of the original developers of the technology, we failed to detect Fab protein capable of binding antigen within the library we generated. Only nonfunctional immunoglobulin heavy and light chain polypeptides were detected. While the phage technology clearly has enormous potential for accessing the diversity of the immune system, the insensitive screening protocols and the low yields of expressed proteins proved to be severe technical limitations in our hands. Recent results from the Lerner lab using filamentous bacteriophage may overcome some of these problems.

Incorrect folding and assembly of the heavy and light chain polypeptides may partially account for the low yields of functional Fab that we obtained in the bacteriophage system. We therefore engineered a smaller, single-chain version of the variable domain of 1F7 (sc1F7) using an idealized linker sequence to connect the heavy and light chains. We utilized Genex's optimized vectors and *E. coli* host strain to overexpress sc1F7. Although we could reproduce the high expression levels obtained previously with an anti-fluorescein single-chain antibody, the yields of sc1F7 were very low. As significant amounts of antibody mRNA were detectable, either translation efficiency is low or, more likely, the single-chain molecule is unstable within the cell. Currently, we are evaluating other linkers, other promoters and other hosts (e.g. protease deficient *E. coli* and yeast) in order to optimize expression.

SIGNIFICANCE: Monoclonal antibodies with tailored catalytic activities and specificities can now be prepared on demand. However, the chemical efficiency of these catalysts is typically considerably lower than that of natural enzymes. Classical genetic selection represents a potentially general method for evolving the properties of these molecules so that they can be used as practical catalysts in research, industry and medicine.

WORK PLAN: Our first objective over the next year is to efficiently express 1F7 in *E. coli* as either an Fab, Fv or scFv protein. This will entail optimization of expression plasmids, host strain, and linker sequence (in the case of the single chain molecule). Literature precedent will guide these experiments. Once an efficient expression system is developed, the catalytic properties of the *E. coli* antibody will be characterized fully. Our second objective is the development of a growth selection assay for 1F7 using a permissive chorismate mutase deficient *E. coli* strain based on KB357 (BioTechnica Inc.). We have already achieved this goal in yeast and similar strategies will be utilized for the bacterial system. Finally, the antibody-encoding genes will be subjected to extensive random mutagenesis and genetic changes that result in improved chemical efficiency of the abzyme will be identified through the selection assay.

#### PUBLICATIONS AND REPORTS:

1. No publications have resulted directly from the studies carried out under the auspices of this grant over the past year. Two manuscripts have been prepared that describe related work, sponsored by NSF, on antibody expression in yeast. Preprints are enclosed.

# ANNUAL PROGRESS REPORT

GRANT # N00014-90-J-1591

R&T CODE 441n047

PRINCIPAL INVESTIGATOR: John M. Stewart

INSTITUTION: University of Colorado Health Sciences Center

GRANT TITLE: Synthetic Helizyme Enzymes

REPORTING PERIOD: 1 July 1990 - 30 June 1991

AWARD PERIOD: 1 March 1990- 31 February 1993

OBJECTIVE: To design and synthesize new kinds of man-made enzymes. To modify the structure of our first designed enzyme, "Chymohelizyme-1," to increase its potency. To study fully the characteristics of these synthetic enzymes.

ACCOMPLISHMENTS (last 12 months): Last July Michael Corey, who had just finished his PhD in enzyme kinetics at Univ. California, Berkeley, arrived to work on the helizyme project. In September we received the stopped-flow spectrophotometer from Applied Photophysics, in England. Mike has applied his much-needed expertise to the project, aided by the stopped-flow instrument, which is very excellent and works well.

DESIGN: In order to study the active site environment of CHZ-1, the synthetic scheme has been redesigned to allow synthesis of analogs with various substituents on the ends of two chains to stabilize further the structure and to provide "reporters" for spectral studies of the topography of this area.

SYNTHESIS: We have had much gratis help from Dr. Kathleen Pugh, a peptide chemist who joined the lab last summer. We lost much time because of difficulty in obtaining suitable synthesis resins. The first CHZ-1 synthesis used the last of a batch of resin we now realize was exceptional, and only now have we found a source for additional amounts of a good resin. We now realize that satisfactory synthesis of this complex molecule requires a resin with exceptional swelling properties, not a problem in synthesis of small peptides. Even with the difficulties we synthesized an analog lacking the His57 residue.

PHYSICAL STUDIES: John Cann, of this department, has shown that CHZ-1 is remarkably stable to denaturation by temperature and chaotropic salts (guanidinium chloride). The midpoint of Gu.HCl dissociation is 2.8 M, as shown by CD. In this midpoint solution, no further dissociation is seen by raising the temperature to 80 degrees. CHZ-1 migrates as a single band in analytical velocity sedimentation. The troublesome dimerization we encountered was found to be due to presence of acetic acid.

ENZYMOLGY: Hydrolysis of carbobenzoxytyrosine nitrophenyl ester (ZTN) by CHZ-1 shows two kinds of catalysis: enzyme-like and general base catalysis. The general base catalysis is fully heat-stable and is seen also with the des-His analog and to a lesser degree with a two-chain fragment bearing the Ser and Asp chains. We



are investigating the mechanism of this effect.

**SIGNIFICANCE:** De novo design and synthesis of an enzyme from basic principles is an important accomplishment. Our work thus opens an entirely new field of research which will undoubtedly have extremely important consequences. Future work in this laboratory and in others will lead to new understanding of enzyme mechanisms and to development of synthetic enzymes designed to catalyze many reactions of medical, defense and commercial importance.

**WORK PLAN** (next 12 months): CHZ-1 will be further studied in detail from chemical, physical and enzymological aspects. A group of anaogs will be synthesized and stustudied to characterize substrate binding and catalytic mechanism. Other physical properties (CD, NMR, Mass spec, crystallography) will be investigated in collaborative studies. Variants with crosslinking between chains 1 and 4 (the Glu and Ser chains) to give further stability will be synthesized.

**PUBLICATIONS:** Paper: Design and synthesis of chymohelizyme-1. J.M. Stewart, K.W. Hahn & W.A. Klis, in "Peptides 1990," E. Giralt and D. Andreu, Eds., ESCOM, Leiden, 1991, pp 574-576.

Abstracts: Amer. Chem. soc. Regional meeting, Indianapolis, IN, May 1991: Helizyme enzymes. J.M. Stewart, J.R. Cann, M.J. Corey, K.W. Han, W.A. Klis.

Amer. Peptide Sympo., Boston, MA, June 1991: Synthetic Helizyme Enzymes: J.M. Stewart, J.R., Cann, M.J. corey, K.W. Hahn, W.A. Klis.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-91-J-1318

R&T CODE: 441g021

PRINCIPAL INVESTIGATOR: Arie Warshel

INSTITUTION: University of Southern California

GRANT TITLE: Computer Simulation of Chemical Reactions in  
Synthetic Model Compound and Genetically  
Engineered Active Sites

REPORTING PERIOD: 1 January 1991 - 30 June 1991

AWARD PERIOD: 1 January 1991 - 31 December 1993

OBJECTIVE: The main objectives of this project is to advance our understanding of biological recognition and specificity by using computer simulation approaches.

ACCOMPLISHMENTS (last 12 months): We have conducted a systematic study of the ability of different simulation approaches to reproduce the energetics of antibody-antigen interaction. This study has established the conditions and the very large cutoff distances (as well as large computer time) needed to obtain reasonable results by Free Energy Perturbation methods. On the other hand, it was found that the microscopic PDL model and its new semimacroscopic version <sup>(1)</sup> provides very practical and extremely efficient way for studies of antibodies.

Other studies <sup>(2-6)</sup> (that were partially conducted under the previous ONR grant) include: (i) The establishment of linear free energy relationships in the action of metalloenzymes <sup>(3)</sup>, (ii) the development of a new microscopic simulation approach that provides a correct estimate of the macroscopic dielectric constants of proteins, (iii) the development of a new dispersed polaron/path integrals approach that allows for the first time direct simulation of quantum tunneling in proton transfer reactions in solutions and proteins <sup>(5)</sup> and new effective ways of incorporating solvent effects in standard quantum chemical programs <sup>(6)</sup>.

SIGNIFICANCE: The PDL and its semimacroscopic versions should allow for a very fast calculation of antibody binding energies. These methods can be incorporated in new interactive docking approaches and provides a starting point for studies of catalytic antibodies. The dielectric constant for the semimacroscopic model can be derived now from a first principle approach <sup>(4)</sup>. The path integral approach can allow one to examine the validity of different enzyme mechanisms by comparing calculated and observed isotope effects.

WORK PLAN (next 12 months): The objectives for the next year include: (i) systematic studies of the catalytic effects of genetic modifications in such systems, (ii) a preliminary study of the role of entropic effects in the catalysis of model compounds. This study is expected to shed some light on the role of entropy in catalysis. (iii) We will study the catalytic activity of synthetic model compound of metalloenzymes.

PUBLICATIONS AND REPORTS (last 12 months):

1. Microscopic Simulations of Chemical Reactions in Proteins and the Role of Electrostatic Free Energy, A. Warshel and J. Aqvist, in Theoretical Biochemistry and Molecular Biophysics, ed. D.L. Beveridge and R. Lavery, Adenine Press (1989) (appears this year) p. 257.
2. Free Energy Relationship in Metalloenzyme - Catalyzed Reactions. Calculations of the Effect of Metal Ion Substitution in Staphylococcal Nuclease, J. Aqvist and A. Warshel, J. Am. Chem. Soc. 112, 2868 (1990).
3. Microscopic Simulations of Macroscopic Dielectric Constants of Solvated Proteins. G. King, F.S. Lee, and A. Warshel, J. Chem. Phys. (1991 in press).
4. Quantum Corrections for Rate Constants of Diabatic and Adiabatic Reactions in Solutions, A. Warshel, Z.T. Chu, J. Chem. Phys. 93, 4003 (1990).

## **BIOPOLYMERIC MATERIALS**

### **CORE PROGRAM**

**SCIENTIFIC OFFICER: DR. MICHAEL T. MARRON**

**PROGRAM OBJECTIVE: TO DETERMINE THE GENERAL PRINCIPLES RELATING MICROSCOPIC THREE-DIMENSIONAL STRUCTURE TO MACROSCOPIC PHYSICAL PROPERTIES OF POLYMERS SUCH AS ELASTICITY, ADHESION, PIEZOELECTRICITY, NONLINEAR OPTICAL PROPERTIES, AND TENSILE STRENGTH IN FIBROUS POLYMERS.**

**NAVY OBJECTIVE: TO DEVELOP A TECHNICAL BASE FOR THE PREPARATION OF NOVEL MATERIALS THAT WILL SERVE NAVAL NEEDS IN MARINE ENVIRONMENTS.**

# ANNUAL PROGRESS REPORT

GRANT #: N00014-91-J-1393

PRINCIPAL INVESTIGATOR: Niels H. Andersen

INSTITUTION: University of Washington

GRANT TITLE: NMR Elucidation of Molecular/Macromolecular Complex Stereochemistry

REPORTING PERIOD: 1 June 1990 → 31 January 1991 ( -88-K-0202 )  
→ 31 May 1991 ( -91-J-1393 )

AWARD PERIOD: → 31 January 1992

OBJECTIVE: To develop methods and software for quantitative analysis of NOESY data and the extraction of experimental constraints for the refinement of structures of biomacromolecules and their complexes as a means for studying molecular recognition phenomena.

## ACCOMPLISHMENTS (last 12 months):

1) The DISCON algorithm for the model-free extraction of accurate cross-rates (and estimates of their experimental precision) from a single NOESY data set has been fully implemented in user-friendly software. The theoretical basis of the method and validation tests are the subject of two submitted papers.

2) The NMR spectra of yeast  $\alpha$  factor in both aqueous glycol and SDS micelle media have been fully assigned.

3) The structure elucidation of endothelin-1, an endogenous vasoconstrictor

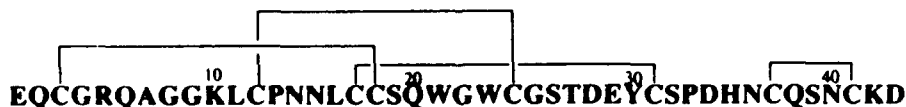
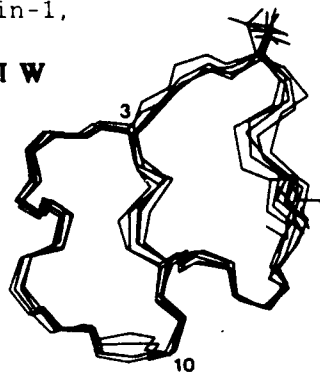
= I/15.3/11-C S C S S L M D K E C V Y F C H L D I I W

has been completed and reported. In it, the major conformer (backbone from 1→15 shown below) was determined to

0.51Å backbone rmsd, and four additional contributing conformers were located. All permissible conformers are  $\alpha$ -helical from K9→C15.

In this conformational mixture, NH  $\Delta\delta/\Delta T$ -values near zero do not correspond to persistent H-bonds.

4) A moderate resolution NMR structure has been obtained for hevein



this is the first solution structure for a member of the agglutinin-toxin fold family of proteins and features- a very short helix from residues 27→32, and an antiparallel  $\beta$  sheet

16)-17-18-19-(20

(26)-25-24-23-22

within a highly constrained core region that extends from N14 to D34.

The backbone structure of the core of hevein is illustrated in the **research highlight** accompanying this report.

SIGNIFICANCE: Until our most recent studies, regions of proteins which were ill-defined in the ensemble of NMR-refinement structures were dismissed as "disordered" and the standard NOESY methodology did not work for smaller peptides that display conformational isomerism. The methods that we are developing should extend the range of systems that can be structurally characterized by NMR to include numerous peptides and the loop dynamics in proteins.

WORK PLAN (next 7 months):

- 1) Complete the characterization of the conformational dynamics of yeast  $\alpha$  factor in free solution and in a micellar association.
- 2) Refine the structure of hevein to greater precision by making pro-R/S assignments and including stereospecific constraints in the refinement. Determine whether the apparent disorder in the loops corresponds to: interconversion of discrete conformers or more random segmental motion.
- 3) Define the pH-dependent changes in conformational dynamics in the endothelin system.

PUBLICATIONS and PRESENTATIONS (last 12 months):

The endothelin structure elucidation was presented in posters or oral presentations at the Spr.'91 ACS Mtg. and at the Keystone Symposium on "Frontiers of NMR in Molecular Biology" (4/91). This structural elucidation has also been featured in numerous guest lectures in which our NOESYSIM/DISCON software was presented.

1) Andersen, N.H., Lai, X., Hammen, P.K., and Marschner, T.M. (1990), Computer-aided Conformational Analysis Based on NOESY Signal Intensities in *NMR Applications in Biopolymers* (Finley et al., Eds.). pp 95-134, Plenum Publ. Co., NY. ( copy enclosed )

2) Andersen, N.H., Lai, X., and Marschner, T.M. (1991), NOESYSIM/DISCON Documentation, copyrighted by Univ. of Washington. (>70 pages, copy available upon request)

3) Krystek, S.R., Bassolino, D.A., Novotny, J., Chen, C., Marschner, T.M., and Andersen, N.H. (1991), Conformation of Endothelin in Aqueous Glycol Determined by 1H-NMR and Molecular Dynamics Simulations, *FEBS Lett.* **281**, 212-218. (recently submitted to the program officer)

\* 4) Rovnyak, G., Andersen, N., Gougoutas, J., Hedberg, A., Kimball, S.D., Malley, M., Moreland, S., Porubcan, M., and Pudzianowski, A., Active Conformation of 1,4-Dihydropyridine Calcium Entry Blockers: Effect of Size of 2-Aryl Substituent on Aryl Rotamer Preference, *J. Med. Chem.*, in press.

\* 5) Lai, X., Chen, C., and Andersen, N.H., Extracting Experimental Distances from NOESY Data: The DISCON Algorithm, an Accurate and Robust Alternative to an Eigenvalue Solution, *J. Magn. Reson.*, submitted.

\* 6) Andersen, N.H., Chen, C., Marschner, T.M., Krystek, S.R., and Bassolino, D.A., Endothelin Conformational Dynamics in Acidic Aqueous Media from Quantitative NOESY Analysis, *Biochemistry*, submitted.

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\* Will be submitted when reprints become available

## ANNUAL PROGRESS REPORT

GRANT#: NO0014-87-K-0387

R&T CODE: 441c-028

PRINCIPAL INVESTIGATOR: Dr. Steven T. Case

INSTITUTION: The University of Mississippi Medical Center

GRANT TITLE: Secretory Polypeptides Encoded in  
Balbiani Ring Genes

PERIOD OF PERFORMANCE 1 JUL 1990 - 30 JUN 1991

OBJECTIVE: To learn about the structure, developmentally regulated synthesis and assembly of a family of secretory proteins (SPs) that are synthesized in salivary glands of an aquatic insect and assemble into an insoluble biopolymer of silk-like threads.

ACCOMPLISHMENTS (Year 04): [1] Identification of additional SP-coding genes. (a) An antibody was raised against a multivalent antigenic peptide (peptide synthesis was done on a core of branched Lys residues) whose sequence was obtained from a partial cDNA clone derived from a 4.8-kb poly(A)+ RNA that is abundant in salivary glands of Chironomus tentans. The antibody reacts specifically with a 185-kDal SP. (2) A cDNA expression library was made to clone the cDNA for a "special secretory protein (sspl60) found in only four cells of Chironomus thummi salivary glands. To screen the library, a polyclonal antibody was raised against gel-purified sspl60. The specificity of the antibody has been confirmed on Western blots. [2] A manuscript was submitted for publication which summarizes the infrared and circular dichroism spectra of synthetic peptides that correspond to "subrepeat" (SR) and "constant" (C) domains of tandem core repeats within spIa, one of the 1000-kDal SPs. Thus far, we have failed to obtain either crystals or laser Raman spectra of these peptides. [3] Antipeptide antibodies were used to search for conserved and diverged epitopes in SPs from different species of Chironomus. Whereas most antipeptide antibodies from C. tentans did not react with proteins from other species, one reacted equally well with sp185 from C. tentans and C. pallidivittatus and sp220 in C. thummi. Oligonucleotides from C. tentans have selected cDNA clones from expression libraries of the other two species.

SIGNIFICANCE: [1] Identification of an additional SP-coding gene in C. tentans will bring us one step closer to describing the primary structure and expression of all members of the SP family in this species. Cloning sspl60 cDNA from C. thummi will enable us to learn about the structure and expression of a unique SP limited to four cells that flank the salivary duct of a C. thummi salivary gland. [2] The structural studies of C and SR peptides suggest that spIs have a unique structure: they are 1000-kDa

fibrous proteins comprised of alternating domains of  $\alpha$ -helix and poly(Gly)II helix. This structure would result in a biopolymer with a unique alternation of contrasting physical properties such as flexibility, extensibility, elasticity and strength. [3] Whereas most SPs are comprised of repeated amino acid sequences with similar motifs, the epitope shared by sp185 in C. tentans and C. pallidivittatus and sp220 in C. thummi is the most conserved structure discovered thus far.

WORK PLAN (Final Months): [1] Complete the sequence new cDNA clones for sp185 in C. tentans. Use anti-ssp160 antibodies to obtain C. thummi cDNA clones encoding this protein. [2] Continue attempts to obtain laser Raman spectra of C and SR peptides. [3] Confirm the identity and begin to sequence cDNA clones encoding sp185 in C. pallidivittatus and sp220 in C. thummi. [4] Write a review on the structure, expression and assembly of SPs in Chironomus.

INVENTIONS: None

PUBLICATIONS AND REPORTS (Year 04):

1. The following paper is in press:

S. T. Case, S. E. Wellman, and S. Hamodrakas (1991)  
Assembly characteristics and structural motifs in an  
aquatic insect's biopolymer. Mater. Res. Soc. Proc.  
(copy enclosed)

2. The following manuscript has been submitted:

S. E. Wellman, S.J. Hamodrakas, E. Kamitosis and S. T.  
Case, Secondary structure of synthetic peptides derived  
from the repeating unit of a giant secretory protein  
from Chironomus tentans. (copy enclosed)

3. The Principal Investigator made oral presentations at:

NSF Biomolecular Materials Workshop, Washington,  
D.C. during 10-12 OCT 1990

Annual Meeting of the Materials Research Society,  
Boston, MA during 2-6 DEC 1990.

4. An Annual Report was distributed on 91 APR 01 to persons  
on the ONR Distribution List and Microbiology/Genetics  
Subset Contractor List.



ANNUAL PROGRESS REPORT.

GRANT #. N00014-90-J-1997.

R&T Code: 441p026---06

PRINCIPAL INVESTIGATOR: John S. Cordingley

INSTITUTION: Univ. of Wyoming, Dept. of Molecular Biology.

GRANT TITLE: The Molecular and Cellular Mechanisms of Quinone Tanning of Proteins.

REPORTING PERIOD: 1 June 1990 - 31 May 1991.

OBJECTIVES: To complete the characterization of the structure of the repetitive schistosome eggshell protein we call F4. To extend our studies of the mechanisms of eggshell formation and to initiate attempts to isolate the secretory vesicles which contain all of the reaction components for the rapid quinone tanning reactions initiated at the time of eggshell formation.

ACCOMPLISHMENTS. The Left-handed alpha-helix. The repetitive eggshell protein F4 has been studied for several years by ourselves leading us to suggest that the basic repeat sequence forms a left handed alpha-helix in solution. This rather unexpected conclusion led us to attempt a number of tests of this hypothesis. The basic repeat is Lys-Tyr-Gly-Tyr-Asp (KYGYD) and computer modelling led us to believe that the left handed helix was stabilized by salt bridges between Lys and Asp side chains. To test this we substituted Asn for Asp in a (KYGYN)<sup>6</sup> peptide. By Circular Dichroism this peptide still had a left-handed conformation but of markedly reduced stability.

We then replaced the glycine residue (the only amino acid with no optical activity) with Alanine but using either D-Ala or L-Ala. The prediction was that the L-Alanine should destabilize the left handed alpha helix whereas D-Ala should stabilize it. This was exactly what we observed. In addition the D-Ala substituted peptide did not adopt a right handed alpha-helical conformation in Trifluoroethanol unlike the glycine containing peptide which readily becomes "right-handed" under these conditions.

In addition to these peptide studies we have extended the computer modelling using different software, namely the CHARM programs. These calculations were carried out by our colleagues at Merck where Russ Middaugh, (Co-PI on our first grant from the Navy) is now located. Essentially this work confirms our original conclusions, i.e. that the left handed alpha helix is the most stable structure. These computer

studies also suggest that substituting Glu for Asp will render the right handed helix the preferred structure. This peptide is being synthesized as I write and the experiments will be done in the next few weeks.

This whole story is now being written up and the manuscripts will be submitted in the very near future.

Rapid Silver Staining of DOPA proteins. We have developed a novel staining method that detects DOPA proteins at very high sensitivity. The staining relies on so-called "Redox-cycling" so that each DOPA molecule actually gives rise to many molecules of silver making the staining very sensitive indeed. This technique is described in the first manuscript listed below.

Phenol-oxidase activity in quinone tanning. In the second manuscript below we show that the phenol oxidase is probably present as a pro-enzyme that is only activated at the time of eggshell formation. In the manuscript we advance a detailed hypothesis for the mode of eggshell formation in schistosomes.

Isolating the secretory vesicles containing the eggshell precursor proteins. One of the primary aims of this grant is to isolate the secretory vesicles containing the eggshell precursors in an untriggered state allowing us to study the triggering process and the reactions in isolation. Our data suggests that all of the reaction components are present in these vesicles so their isolation is the key to further progress. The isolation is proving difficult as we thought it might and attempts continue. We remain optimistic that we can find a way to isolate these vesicles and we will keep you posted.

#### PUBLICATIONS.

Wells, K. and Cordingley, J.S. (1991). Detecting proteins containing 3,4-Dihydroxyphenylalanine by silver staining of polyacrylamide gels. *Analytical Biochemistry* 194, 237-242.

Wells, K. and Cordingley, J.S. (1991) "Schistosoma mansoni: Eggshell formation is regulated by pH and Calcium" in Press in *Experimental Parasitology*.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-91-WA-24255

R & I CODE: 441P022---05

PRINCIPAL INVESTIGATOR: Bruce Paul Gaber

INSTITUTION: Naval Research Laboratory

GRANT TITLE: Molecular Modeling of Lipid Bilayers

REPORTING PERIOD: 1 July 1990-30 June 1991 (12 months)

AWARD PERIOD: 1 July 1990-30 June 1991

OBJECTIVE: We are applying computer-aided molecular modeling techniques to the study of lipids and the microstructures which they form.

ACCOMPLISHMENTS: *Software Development*--The molecular modeling program NanoVision, for which initial developmental work was done under this project, is now being published and distributed by the American Chemical Society. We have written a program (Bilayer Builder) which allows us to build a lipid bilayer of any arbitrary dimension given the fractional coordinates, unit cell dimensions and space group for the crystal. We have adapted the ribbon motif, widely used to display protein secondary structure, to the depiction of the acyl chains of phospholipids. This tool has allowed us to determine that, contrary to the conventional wisdom, acyl chains are not universally planar all-trans structures. Rather, many chains exhibit systematic twists, bends, and bows. *Bioinformatics*--As part of our overall effort in molecular modeling, we have become increasingly involved in development of tools for extracting specific biophysical information from large databases. We have recently completed work on NRL\_3D, a sequence-structure data base which links the National Biomedical Research Foundation's (NBRF) Protein Identification Resource (PIR) sequence database with Brookhaven National Laboratory's Protein Data Bank (PDB). NRL\_3D permits PIR sequence search tools to be used on the PDB structural data. Thus a specific sequence, or an incomplete sequence, may be specified and the structures corresponding to that sequence retrieved from PDB and displayed. NBRF now includes a copy NRL\_3D with all releases of PIR. Unfortunately for lipid chemists, no database comparable to PIR or PDB exists for lipid structures. We have begun to deal with this deficiency with the development of NRLipid, a HyperCard database of lipid structural information. NRLipid consists of cards for each of the phospholipids for which crystallographic data is available. Basic information such as nomenclature, reference, space group and unit cell dimensions, occupy one card. A second card contains the actual crystallographic fractional coordinates for the molecules of the asymmetric unit. We have further refined the simple lipid organizational scheme we call the Hierarchy of Lipid Assembly. The preliminary scheme distinguishes three levels of lipid interaction: alpha, beta and gamma. Each level is defined by characteristic molecular dimensionality and length scale for interaction: Alpha interactions are one dimensional and short range; Beta interactions are two dimensional, occurring on what physicists refer to as the meso-scale; Gamma interactions are three dimensional and long (on a molecular scale). The Hierarchy describes examples of the interaction, the forces governing the interactions, and their structural consequences. *Consensus Modeling*--In earlier reports we have described the development of what we have termed "consensus models"--molecular models constructed in the absence of crystallographic data, but rather using data derived from other compelling biophysical measurements. As part of our effort to study lipid-protein interactions, we have extended the consensus modeling approach to forming molecular models from the "quasi-models" derived by Jacobs and White (Biochem (1989) 28, 3421-3437) from their neutron and X-ray scattering data. The construction of the models began with the

construction of a computer model of dioleoylphosphatidylcholine (DOPC). The DOPC model was relaxed using energy minimization. The DOPC model was next compared to a "ruler" constructed from the Jacobs and White data which partitioned various portions of the DOPC molecule. The DOPC was positioned within the framework established by the ruler. The final positioning weighted by confidence that the experimentalist imparted to each partition. Once a two dimensional representation of DOPC was achieved, water and a simple hydrophobic peptide studied by Jacobs and White was added to the model. The result is a simple, but reasonably accurate, molecular graphics representation of the complex of lipid, peptide and water.

**SIGNIFICANCE:** We have extended molecular graphics techniques, formerly the exclusive domain of protein and nucleic acid research, to the study of lipids. Our research has resulted in the development of methods for the generation, manipulation, refinement, and depiction of molecular models of individual lipids and their microstructures.

**WORK PLAN (next twelve months):** We have begun studies of the interaction of a series of hydrophobic di- and tripeptides with single component lipid bilayers. These peptides have been shown to inhibit the fusion of Sendai virus with cells. Further these same peptides (such as Z-Phe-Phe-Gly, Z-Phe-Tyr and Z-Gly-Phe) inhibit the fusion of N-methyl DOPE lipid vesicles. Our goal is to collect experimental data with which to test various possible molecular models of interaction of these peptides with lipid bilayers, and to deduce the mechanism by which vesicle fusion is inhibited.

The experimental work consists of determining association constants for peptides with lipid vesicles. In collaboration with the Biocalorimetry Center, Johns Hopkins University, we are determining these constants by differential titration calorimetry. From this data we can readily calculate the free energy of association. These free energy data then become the experimental data against which we will test our models. The models will be various actual molecular configurations on which we will conduct free energy perturbation calculations. The free energies determined experimentally will be compared with those calculated for each of the potential models to determine which, if any, of the models yields comparable free energies. The experimental work is being done by Dr. David Turner, an NRC Research Associate in our group. The free energy perturbation calculations are under the direction of an ONR Young Investigator, Prof. Kenneth Merz of Pennsylvania State University, with whom we have established a formal collaboration.

**PUBLICATIONS AND REPORTS (last 12 months):**

1. Namboodiri, K., Pattabiraman, N., and Gaber, B. P. (1990) NRL\_3D, a sequence-structure database searchable within PIR. Prot. Seq. Anal.
2. Light, W. R. and Gaber, B. P. (1991) NanoVision--molecular graphics for the Macintosh. J. Mol. Graphics (submitted 6/91).
3. Turner, D. C. and Gaber, B. P. (1991) Bilayer Builder. J. Mol. Graph. (subd 6/91).
4. Hybl, A. and Gaber, B. P. (1991) Adaption of the ribbon algorithm to lipid depiction. J. Mol. Graphics (submitted 6/91).
5. Namboodiri, K., Chen, J., and Gaber, B. P. (1991) NRLipid--A hypercard database for lipid structures. Abst. Amer. Cryst. Assoc. Cleveland, July, 1991.
6. Gaber, B. P., Namboodiri, K., Turner, D. C., and Hybl, A. (1991) Lipids--a new view. in Biomembrane Structure and Function--the State of the Art, B. P. Gaber, and K. R. K. Easwaran, eds. Adenine Press, Albany, NY. (in press).
7. Gaber, B. P., Namboodiri, K., Light, W. R., Turner, D. C., and Hybl, A. (1991). Tools for molecular modeling of lipids. in Membrane Biotechnology, J.-C. Gomez, Ed. Wileylausser (in press).

## Final Report

GRANT #: N00014-88-K-0388

PRINCIPAL INVESTIGATOR: Pui Shing Ho

INSTITUTION: Oregon State University

GRANT TITLE: DNA mediated electron transfer and application to "Biochip" development

REPORTING PERIOD: 1 June, 1988 - 31 May, 1991 (3 Years)

AWARD PERIOD: 1 June, 1988 - 31 May, 1991

OBJECTIVE: To study the electronic properties of double-stranded DNA as to determining whether this macromolecule can support electron transport processes. This pertains to possibly utilizing the base sequence and secondary structure of DNA as a matrix for developing molecular level electronic components.

ACCOMPLISHMENTS (3 years): We have successfully synthesized a number of porphyrin and acridine derivatives for direct measurement of electron transport through DNA. We are currently determining the steady-state reduction potentials of the modified DNAs to assess the utility of these as photoinducible electron donors and acceptors. We have also completed and tested the flash photolysis apparatus for the direct absorption and emission measurements of electron transfer rates. The primary drawbacks in the progress of this project have been the difficulty in the synthesis of the modified DNAs, and in assembling a working flash photolysis apparatus. Both problems have been solved or circumvented and we are now ready to perform the actual electron transfer measurements.

In related studies, the electronic properties of DNA single crystals were recorded (in collaboration with Prof. L. Clark, Department of Chemistry, UCSD), and compared for d(CG) and d(UA) base pairs in Z-DNA by recording the polarized reflectance spectra of d(m<sup>5</sup>CG)<sub>3</sub> and d(m<sup>5</sup>CGUAm<sup>5</sup>CG) single crystals. These studies show that the electronic coupling between the aromatic bases of DNA duplexes are highly sequence specific. The sequence containing only d(CG) base pairs showed much greater resolution of the two major  $\pi$ - $\pi^*$  transitions that are characteristic of the Z-conformation. We are currently in the process of comparing the polarized absorbance spectra of a number of other DNA sequences and conformations. In one study, we plan to use the UV absorption spectrum and its linear dichroism of a DNA sequence in an unsolved crystal to determine the conformation and orientation of the oligonucleotide in the unit cell. This would be an attempt to utilize the spectroscopic properties of a crystal to help in solving the phase problem associated with determining the structure of the DNA.

We have solved the structures of two oligonucleotide sequences in the presence of copper(II) ions, and shown that this metal binds in a covalent manner to the guanine bases of double stranded DNA. The binding is base specific. Only purines are bound and, of the purine bases, only guanines are consistently modified. The binding of copper (II) to adenine bases must be facilitated by additional intermolecular

interactions, and would not be expected to occur with DNA in free solution. Thus double stranded DNA crystals can be modified, or 'doped', in a base specific manner to affect the electronic properties of the crystal.

In other related work, we have developed and characterized a theoretical criteria for predicting the relative stability of various DNA sequences as Z-DNA in solution and, from this, predicting the solution conditions for the crystallizing hexanucleotides as Z-DNA. The method uses solvent accessible surface calculations to estimate the relative stability of the hexanucleotides in the Z-form versus the B- and single-stranded conformations. This lead to a prediction for the driving force required to induce formation of the left-handed conformation in solution and, when coupled with information on the intrinsic solubility of the conformation, leads to a prediction for how to crystallize Z-DNA. We applied this to the sequence d(CICGCG), where the base I is deoxyinosine, and found that the method predicted exactly the conditions at which Z-DNA crystals formed. When we examined the energetics for the packing of Z-DNA hexamers in the crystal lattice, we found that the lowest energy packing of the hexanucleotides was that observed for the actual crystals. The energy for packing resulted from van der Waal's contacts that overcome the inherent conformational entropy and electrostatic repulsion of the DNA in the crystal.

SIGNIFICANCE: The ability to predict the crystallization of Z-DNA hexanucleotides is significant in that we can now consider the process of crystallizing biologically interesting macromolecules as a science rather than a random set of events. This will have applications towards the ability to crystallize other oligonucleotides in different conformations, and relates to the general process of macromolecular crystallization for x-ray diffraction studies.

The base dependence of the polarized reflectance spectra from of DNA in single crystals shows that the electronic properties of the DNA polymer are attenuated by base composition.

WORK PLAN (next 12 months): This is the end of the funding period for this contract in our laboratory. Our plans, however, are to continue to work in this area, with the next twelve months devoted to actual measurements of electron transfer processes, after the arduous task of assembling the molecular systems and the experimental apparatus. Furthermore, we plan to continue the measurement of the electronic spectra of other DNA single crystals, varying the base composition and the conformation (to the B-form) of the oligonucleotides. Finally, we plan to extend the working hypothesis for Z-DNA crystallization towards B-DNA and A-DNA crystallizations.

PUBLICATIONS AND REPORTS FROM WORK SUPPORTED BY THE ONR (3 years):

- Kagawa, T.F., Stoddard, D., Zhou, G., and Ho, P.S. (1989) "A quantitative analysis of DNA secondary structure from solvent accessible surfaces: The B- to Z-DNA transition as a model", *Biochemistry*, **28**, 6642-6651.
- Zhou, G., and Ho, P.S. (1990) "Stabilization of Z-DNA by demethylation of thymine bases: 1.3 Å single crystal structure of d(m<sup>5</sup>CGUAm<sup>5</sup>CG)", *Biochemistry*, **29**, 7229-7236.
- Ho, P.S., Zhou, G., and Clark, L.B. (1990) "Polarized electronic spectra of Z-DNA single crystals", *Biopolymers*, **30**, 151-163.
- Green, S.A., Simpson, D.J., Zhou, G., Ho, P.S., and Blough, N.V. (1990) "Intramolecular quenching of excited singlet states by stable nitroxyl radicals", *J. Am. Chem. Soc.*, **112**, 7337-7346.
- Daniels, M., Hart, L.P., Ho, P.S., Ballini, J.-P., and Vigny, P. (1990) "Time-resolved spectroscopy of the intrinsic fluorescence of nucleic acid species", *SPIE Proc.*, **1204**, 304-313.
- Kagawa, T.F., Wang, A.H.-J., Stoddard, D., and Ho, P.S. , "Covalent modification of guanine bases in DNA by copper (II) ions: 1.2 Å crystal Z-DNA structure of CuCl<sub>2</sub> soaked d(CG)<sub>3</sub>", in press, *J. Biol. Chem.*
- Geistranger, B., Kagawa, T.F., Quigley, G.J., and Ho, P.S. "Sequence specific modification of purine bases in DNA by copper (II) ions: 1.3 Å crystal Z-DNA structure of CuCl<sub>2</sub> soaked d(m<sup>5</sup>CGUAm<sup>5</sup>CG)", in press. *J. Biol. Chem.*
- Ho, P.S., Kagawa, T.F., Tseng, K., Schroth, G., and Zhou, G., "Prediction of a crystallization pathway for Z-DNA hexanucleotides", Submitted for publication.
- Tseng, K., Kagawa, T.F., and Ho, P.S. "Effect of minor groove substituent groups on the stability of Z-DNA", in preparation.

## ANNUAL PROGRESS REPORT

GRANT#: N00014-91-J-1455

R&T PROJECT: uri5303-9101

PRINCIPAL INVESTIGATOR: C. Anthony Hunt

CO-INVESTIGATOR: Roderick D. MacGregor

INSTITUTION: University of California, San Francisco

GRANT TITLE: Synthesis of a Self-Assembling Supramolecular Lattice

REPORTING PERIOD: 01 May 1991 - 01 June 1991

AWARD PERIOD: 01 May 1991 - 30 April 1994

OBJECTIVE: The goal of this project is to construct a self-assembling, closed, hollow, uniform, supramolecular lattice in which the lattice dimensions measure between 10 and 100 nm, and which exhibits two hierarchical levels of molecular order. Our approach is novel. Use the membrane skeleton of erythrocytes, which is an ordered, two dimensional molecular lattice, as a template to construct a synthetic lattice that remains intact, functional and responsive after removal of the template.

ACCOMPLISHMENTS: In the one month this project has been funded we have initiated work on the project, refined our initial experimental approach, begun evaluating options for the first hub molecule, and have interviewed and hired a Post Doctoral Scientist to work on the project starting in July.

SIGNIFICANCE: If this project is successful we will gain basic knowledge of cell membrane structure, function and assembly, and will construct a synthetic, self-assembling, closed, hollow, uniform, supramolecular lattice. The lattices are expected to possess unique materials properties.

WORK PLAN (next 12 months): Work on this project is just beginning. The first objective is to contrast the observed and model-predicted binding of model hub molecules to normal erythrocytes and to erythrocytes having abnormal membrane skeletons. This will require the preparation and purification of at least three candidate hub molecules. The candidate hub-forming molecule which best exhibits the quantal behavior predicted by our newly developed corralled membrane model will be selected to become the hub-forming molecule.

PUBLICATIONS AND REPORTS (last 1 month): None.



## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-3001

R&T CODE: 4412068

PRINCIPAL INVESTIGATOR: Sangtae Kim

INSTITUTION: University of Wisconsin - Madison

GRANT TITLE: Microstructural Models of Interactions that Govern Protein Conformations

REPORTING PERIOD: 1 June 1990 - 30 June 1991

AWARD PERIOD: 1 July 1989 - 30 June 1991

OBJECTIVE: To apply computational methods for microstructures suspended in a viscous fluid to the calculation of the slower dynamic processes in protein folding. The concerted motion of the entire tertiary structure will be considered with the secondary constituents modeled as solid segments.

ACCOMPLISHMENTS(last 12 months): Diffusion coefficients were computed for Lysozyme and BPTI. We are now developing our own boundary element mesh generation program. After having completed the interfacing of the Brookhaven Molecular Surface Package created boundary elements that were too "skinny" and created problems for smaller proteins like BPTI. We lost about three months chasing this down (a disappointment).

We developed a new method that speeds up the iterative solution by incorporating information from other approximate hydrodynamic solutions. The CDL-BIEM velocity representation consists of two parts: a (hydrodynamic) double layer potential, and a so-called range completer which is responsible for representing the net force and torque on the particle. The simplest examples of the latter are point force (Green's function) and point torque solutions to the Stokes equations. But we can do better. In the mobility calculations, instead of distributing the known external force and torque at a single point, we distribute these a priori using physical insight or known approximate singularity solutions.

SIGNIFICANCE: The significance of predicting protein diffusion coefficients using CDL-BIEM lies in the fact that we will be able to test the Stokes-Einstein theory without making any assumptions on how to calculate the mobility of the rigid bodies we use to model the proteins. Past calculations of diffusion coefficients have relied on three sets of assumptions.

- 1) Assumptions associated with the Stokes-Einstein Theory.
- 2) Assumptions on how to generate an appropriate

molecular surface (using the given atomic coordinates) to use in the theory.

- 3) Assumptions on how to calculate the mobility of the rigid body defined by the molecular surface.

We can eliminate the third set thus shedding light on the first two and gain insights on more complicated problems such as dynamics of internal motions.

WORK PLAN (next 12 months): The objectives can be divided into three fronts.

1. Continue mapping the CDL-BIEM algorithm onto the most likely candidates for future high-performance parallel architectures. These include:

- a. (Christine Maul) The 16-processor Intel iPSC/860 recently acquired by the Center for Parallel Computational Engineering, UW-Madison.
- b. (Brian Saunders) The CM-2 and successor(s). Since a significant portion of CDL-BIEM is dataparallel, we will continue to work with the Connection Machine.
- c. The Computer Sciences Department PRISM project. A \$2M budget to purchase the state-of-the-art in MIMD or SIMD machine, FY 91-92.

2. (Gary Huber) Interactions between secondary structures.

3. (Doug Brune) Simulate interactions between two proteins.

PUBLICATIONS AND REPORTS (last 12 months):

Pakdel, P. and Kim, S. (in press) Mobility and stresslet functions of particles with rough surfaces in viscous liquids. J. Rheology, 35.

Fuentes, Y.O., and Kim, S. (1991) Foundations of Parallel Computational Microhydrodynamics: Communication Scheduling Strategies, **Rheology Center Report #127**.

Kim, S. and Karrila, S.J. (1991) Microhydrodynamics: principles and selected applications, Butterworth-Heinemann, Boston.

Kim, S. and Brune, D.A. (1990) Theoretical predictions of translational and rotational diffusion coefficients of proteins. Molecular Biophysics of Proteins, Peptides and Polynucleotides, AIChE National Meeting.

Kim, S., Brune, D.A., Huber, G., and Pakdel, P. (1990) Computer visualization techniques for colloidal systems, particle mechanics, and protein dynamics. Visualization of Chemical Engineering Systems, AIChE National Meeting.

Kim, S. (1991) Invited Talk: Diffusion and mobility of proteins: hydrodynamics of protein structures. Bioseparations, Bioanalysis and Biocatalysis, Midwest Biotechnology Symposium, 1991.

## ANNUAL PROGRESS REPORT

GRANT#: N00014-91-J-1457

R&T CODE: uri5304-9101

PRINCIPAL INVESTIGATOR: Scott A. Lee

INSTITUTION: University of Toledo

GRANT TITLE: Synthesis of Novel Composite Platinum-DNA  
Films via Wet-Spinning

REPORTING PERIOD: 1 March 1991 - 30 June 1991

AWARD PERIOD: 1 March 1991 - 28 February 1994

OBJECTIVE: The primary objective of this project is to synthesize novel composite films consisting of DNA intercalated with platinum-based compounds via the wet-spinning technique. Another objective will be to synthesize films of hyaluronic acid with different metallic counterions via the wet-spinning techniques. The acoustical and optical properties of these films will be evaluated via Raman scattering, Brillouin scattering, measurements of the refractive indices and optical polarizabilities, second harmonic generation experiments, x-ray scattering, optical microscopy, and, gravimetric measurements.

ACCOMPLISHMENTS (last 3 months): I have completed the detailed design of the wet-spinning apparatus. As part of this process, I had to consider the optimum design of the spinnerets (material, thickness, the number, spacing, and diameter of the holes for extrusion), the best height for the spinning column (determined by the rate at which the entire DNA bundle is precipitated by the ethanol bath), the spinning drum (material and diameter), and the mechanism for controlling the rate of spinning as well as translating the drum. With these parameters fixed, I have ordered the appropriate syringe pump for extruding the DNA through the spinneret, the spinnerets, the glassware, and the stepper motors (to control the spinning process) from commercial companies. Work orders have been submitted to our machine shop to construct special parts involving bearings (for use in the rod controlling the spinning drum and the water-tight seal into the bathing solution). The guiding slot necessary to feed the DNA fiber onto the spinning drum has been designed and constructed. We have begun the assembly of the wet-spinning apparatus. I should point out that, though the starting date for this grant was 1 March 1991, due to delays, the money only became available for me to use here at the University of Toledo in early May.

SIGNIFICANCE: Earlier studies of wet-spun films of DNA have shown that the acoustical and optical properties of these

films depend greatly on the conformation of the DNA molecule. Dramatic changes in the conformation can be induced by intercalating the DNA with platinum compounds. We will produce such films via the wet-spinning process and evaluate their acoustical and optical properties. It is expected that the intercalation process will produce films of **enhanced** acoustical and optical properties.

WORK PLAN (next 12 months): The objective of the next year's work is to complete the wet-spinning apparatus, produce initial films of pure DNA (to verify that the apparatus is working properly), produce intercalated Pt-DNA films as well as hyaluronic acid films, and to perform initial experiments to evaluate the optical and acoustical properties of these films.

PUBLICATIONS AND REPORTS (last 3 months): Since this work has only just started, there have not been any publications or reports to date. However, we will give a talk about the wet-spinning apparatus at the 1991 Fall Meeting of the Ohio Section of the American Physical Society in October, 1991. Further reports and articles from this work will follow shortly.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-86-K-0369

R&T CODE: 441p013

PRINCIPAL INVESTIGATOR: R. W. Lenz and R. C. Fuller

INSTITUTION: University of Massachusetts

REPORTING PERIOD: 1 July 1990 - 30 June 1991

AWARD PERIOD: 1 November 1986 - 28 February 1993

OBJECTIVE: The objective of this project continues to be a multidisciplinary study of the biosynthesis, regulation and material properties of microbial poly- $\beta$ -hydroxyalkanoates (PHAs). Specifically we are proceeding to determine and optimize, the biochemical variables that regulate polymer production; to induce a variety of microorganism to synthesize, new unnatural and functional PHAs by regulating substrates and environment; and to further characterize the physical, chemical, and material properties of these polymers.

ACCOMPLISHMENTS: During the current funding year we have completed critical experimental work in three major areas of the research project.

1. The primary organism used was the phototrophic bacterium *Rhodospirillum rubrum* which shows great versatility in producing unique PHAs. It has deficiencies in being a slow grower and produces low yields of PHAs. *R. rubrum* is known to grow under two general culture conditions, either anaerobically in the light or aerobically in the dark. All results presented have employed anaerobic conditions. Polymer yield was generally increased if aerobic conditions were employed, but the resultant polymers were generally found to be solely P(4HB-co-HV) copolymers. Olefin and deuterium incorporation can be obtained in the pendent group of the PHAs produced by this bacterium. The use of a co-fed substrate allowed the incorporation of the desired monomer unit. When  $\gamma$ -hydroxybutyric acid is co-fed with butyric or valeric acid, the resultant polymer seems to contain a substantial amount of hydroxymethyl pendent groups with very little 4HB incorporation. However when  $\gamma$ -hydroxybutyric acid is co-fed with caproic acid, the resultant polymer contains a large fraction of 4HB (20 mole %). Hopefully, co-feed systems can be developed which lead to synthesis of desired and useful bacterial polyesters.

2. During the past year we have also focused on the physiology of *Pseudomonas oleovorans* grown on octanoic acid. Our goals were to determine the optimal growth conditions for the production of PHO (poly-3-hydroxyoctanoate) and to establish criteria which would maximize the product yields of batch cultures.

Large amounts of polymers were obtained using closely monitored culture conditions. Nitrogen limitation, accompanied by a carbon excess, decreased the metabolic rate and increased the total polymer yield up to 10-fold. Further cultivation exhausted the carbon source and a very rapid decrease in the PHA content of the cells. Addition of the carbon source prevented or reversed PHA degradation. During growth the percent composition of the repeating monomer units remained constant in the PHO produced.

The conditions established in this study were applied to 12 L cultures, and after an initial 12 hour growth period, the culture was supplemented at two hour intervals with 10 mM sodium octanoate for an additional 12 hours. Under these conditions PHA was produced at quantities approaching 5 grams per liter. The products of these cultures are being used for determination of the physical and mechanical properties of the material.

3. Using *Pseudomonas oleovorans* to synthesize PHO, sufficient quantities were produced (using techniques described in the last ONR report) to confirm batch consistency via GC, DSC, GPC, and TGA experiments. Sample preparation techniques were explored and mechanical testing and thermal evaluations were conducted on the thermoplastic elastomeric PHO. In addition, commercially available thermoplastic elastomers (TPEs) were obtained and tested to insure that the techniques developed resulted in data consistent with published data and to determine how the mechanical properties of the bacterial polyester compare to other TPEs.

The following results for PHO were obtained:

Mechanical properties (all within Properties the range of commercially available TPEs)		Thermal	
Young's modulus (E)	7.5 MPa	TGA: T <sub>onset of</sub>	237°C
tensile strength	12 MPa	weight loss in	
elongation at break	350%	nitrogen	
hardness	60 Shore A	DSC: T <sub>m</sub>	55°C
	37 Shore D	T <sub>g</sub>	-35°C
tensile set @ 100% strain =	35%	ΔH <sub>m</sub>	15 J/g

#### SELECTED PUBLICATIONS AND REPORTS (7 of 10):

K. Fritzsche, R.W. Lenz and R.C. Fuller, "An Unusual Bacterial Polyester with a Phenyl Pendant Group", Makromol. Chemie., 191, 1957-1965 (1990).

A. Ballastrieri, G. Montaudo, G. Impallomeni, R.W. Lenz, Y.B. Kim, and R.C. Fuller, "Sequence Distribution of  $\beta$ -Hydroxyalkanoate Units with Higher Alkyl Groups in Bacterial Copolyesters", Macromolecules, 23, 5059-5064 (1990).

R.W. Lenz, B.-W. Kim, H.W. Ulmer, K. Fritzsche, E. Kneé and R.C. Fuller, "Functionalized Poly- $\beta$ -hydroxyalkanoates Produced by Bacteria" in E.A. Davies, Ed., "New Biosynthetic Biodegradable Polymers of Industrial Interests from Microorganisms", Proceedings of the 1990 NATO Workshop in Sitges, Spain, Kluwer Academic Publishers, The Netherlands, 1990, pp. 23-35.

M. Wolf, E.J. Kneé Jr., R.W. Lenz and R. C. Fuller, "Influence of Growth Conditions on Production and Composition of Poly- $\beta$ -hydroxyalkanoates by *Pseudomonas oleovorans*", Applied. Environ. Microbiol. (in press)

M. Liebergesell, E. Hustede, A. Timm, A. Steinbuckell, R.C. Fuller, R.W. Lenz, and H.G. Schlegel, "Formation of Poly( $\beta$ -hydroxyalkanoates) by Phototrophic and Chemolithotrophic Bacteria", in Archives of Microbiology (in press 1991).

M. Beinvenuti and R.W. Lenz, "Polymerization and Copolymerization of  $\beta$ -Butyrolactone and Benzyl- $\beta$ -Malolactonate by Aluminoxane Catalysts", J. Polymer Sci., Part A: Polymer Chem., 29, 793-805 (1991).

R.W. Lenz, H. Ulmer, Y.-B. Kim and R.C. Fuller, "Production of Non-Natural and Functional Poly- $\beta$ -hydroxyalkanoates by Bacteria" in "Contemporary Topics in Polymer Science, Volume 7", J. Riffle and J.C. Siamone, Eds., Plenum Publishing Corp., New York, N.Y., 1991.

## PROGRESS REPORT

Grant no. N00014-89-J-1564

PRINCIPAL INVESTIGATOR: Randolph V. Lewis

CONTRACTOR: University of Wyoming

CONTRACT TITLE: Cloning and Structure of Spider Silk

PERFORMANCE PERIOD: July 1, 1990 - May 15, 1990

RESEARCH OBJECTIVES: Clone, sequence and express dragline silk protein from Nephila Clavipes and compare the sequence to clones of the same protein from Areneus gemmoides.

PROGRESS: The major data on our previous protein(silk protein 1) are summarized in the publications in PNAS and in ABB. That data indicated to us that a second protein must be present in the dragline silk fiber because we could not find the sequence of one major peptide we had isolated. Therefore we went back and screened our original library with a probe for that peptide. We have sequenced over 2 kb of two separate clones for the second Nephila dragline silk protein(silk protein 2). One of the clones has the poly A tail and thus we have the 3' end of the mRNA in that clone. From this data we have compiled the repeating protein segment. It is clear that the repeats are not exact although common features are present. There are three regions to the repeat as we saw in protein 1. The first is highly conserved in both length and sequence. The sequence is GPGQQGPGGYGPGQQGPGSGPGS. The second consists of (A)<sub>x</sub> and is 7 to 10 amino acids long. This segment is well

conserved in all repeats with only an occasional Ser substitution. The third is poorly conserved consisting mainly of repeats of GPGGY and GPGQQ and is up to 20 amino acids in length. There are several deletions but they are all multiples of the 5 amino acid repeat. This is exactly what was seen in silk protein 1 except the repeats were 3 amino acids and deletions were multiples of those.

An interesting sidelight is the DNA codon usage. There is a very strong bias against the use of G and C in the third position. This is seen in most of the amino acids to the extent that some have only 3-5% of the codons with G or C at the wobble base. This is exactly the same as was seen in silk protein 1.

We have inserted the longest clone for each protein into an expression vector and sequenced the vector to insure that the insert is in frame. Expression of protein seems to be, if not lethal, certainly not conducive to growth. Thus, we find the cultures soon are predominantly deletion mutants in the plasmid. We do get detectable expression via Coomassie stained gels but it is still fairly low. We are in the process of trying a baculovirus system which we feel may give us much better protein production.

We have synthesized peptides corresponding to the complete repeat segment and to the third, highly conserved, region of silk protein 1 and to the complete repeat of silk protein 2. These are being used in biophysical studies primarily with CD and NMR. The peptides of protein 1 show significant similarities to the silk protein itself. The 30P peptide(which is the total repeat segment) appears to be a triple strand helix at low temperatures based on the CD data. As the temperature is increased to 80°C this is converted to a clear  $\beta$ -sheet structure. This is concentration dependent as it occurs above 2 mg/mL. Below that concentration the peptide shows only an unstructured form. The same peptide when placed in TFE to determine ability to form helices, shows significant helix formation between 20 and 40% TFE. The CD is identical to a poly-Ala helix. The 15P peptide(which is only the (GGX)<sub>n</sub> region)

also has the same concentration and temperature characteristics as 30P. However, in TFE there is no evidence of helix formation. Some of this data is presented in the ABB paper. The peptide for protein 2 has been purified and is currently being studied. In addition we have initiated a collaboration with Art Pardee at Colorado to use 2-D NMR to study the structure of both sets.

Based on the data from protein 1 we proposed a mechanism for elasticity and strength last year. The strength is due to interprotein chain hydrogen bonds from the  $\beta$ -sheet regions. These are spaced such that the proteins are overlapping at different points in the chain. The elasticity is due to the poly-Ala regions that form a helix when stretched which is entropically unfavorable and when the tension is released returns to a disordered state.

The second protein has forced us to significantly alter this mechanism. The second protein cannot form a typical  $\beta$ -sheet. Instead it appears to form a series of linked  $\beta$ -turns. If these were then to stack together the FTIR and probably the X-ray diffraction would appear like  $\beta$ -sheet since in one direction that is what it would be just not in an extended form. The elasticity would then be due to both the helix forming regions and the linked turns which would stretch. Both would then relax to their original structure. The CD data on protein 1 in the 20-40°C range could be interpreted as linked  $\beta$ -turn type 2. The studies on the solubilized silk protein also are consistent with this type of structure. In order to better study this we are setting up a collaboration to use fiber X-ray diffraction to look at this possible mechanism.

**SIGNIFICANCE:** The structure of a second protein comprising spider dragline silk has been determined and it provides the basis for a mechanism to explain the mechanical properties of dragline silk fibers.

**WORK PLAN**(year 5): The work to express these two proteins will continue with the expected result being a system we can use to express the protein and variants we hope to construct. It is likely we will have to turn to a baculovirus system to achieve this. We will continue our studies of the peptides we have synthesized by using NMR and FTIR to confirm the structures suggested by the CD work. We will also make polymers of them in order to study the higher order structure. We should have enough sequencing completed soon to insure that we have the proper *Areneus major* ampullate silk and minor ampullate silk clones and begin to identify the repeated segments of that silk. We expect to be well on our way to confirming or destroying our mechanism during this period.

**INVENTIONS:** The patent filed last year was updated to include the new protein and the fact that both proteins are needed for dragline silk fiber formation. In addition the patent for both proteins has been filed in Europe and in Japan.

#### **PUBLICATIONS:**

1. Dong, Z., Lewis, R.V. and Middaugh, C.R. (1991) Molecular Mechanism of Spider Silk Elasticity. ABB 284:53-57.
2. Xu, M. and Lewis, R.V. (1990) Structure of a Protein Superfiber: Spider Dragline Silk Protein. PNAS 87:7120-7124.
3. An oral presentation was given combining these two papers and the new protein data at the ACS meeting in Atlanta. At that meeting numerous written and radio interviews were given. The follow up to this year's and last year's presentations have been several popular magazine articles as well as newspapers articles world-wide.



## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1455

R&T CODE: 44lpin1

PRINCIPAL INVESTIGATOR: Stuart Lindsay

INSTITUTION: Arizona State University

GRANT TITLE: Biological Applications of STM and AFM in Water

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 January 1990 - 31 December 1992

OBJECTIVE: To improve methods for imaging biological polymers by scanning probe microscopy in an aqueous environment.

ACCOMPLISHMENTS (last 12 months): We have completed a systematic study of the electrochemistry of nucleic acids (and their constituents) on gold electrodes (our STM/AFM substrate of choice). This allows us to understand the irreversible reactions that have been used to 'pin' nucleic to substrates for imaging.

A new imaging method was developed. The method is based on retaining the molecules on a surface *under potential control* so that molecular adsorption is reversible. Both STM and AFM images were formed with the new method. We have improved substrate preparation methods and control of contamination to the point where we see the well known (in UHV) Au(111)- $22 \times 3^{1/2}$  reconstruction *routinely* on the electrode surface *in situ*. This has not been observed at a liquid-solid interface before.

We have measured current-voltage and current-distance curves over a number of molecular adsorbates as part of an effort to further understand contrast in the STM. We have used *in situ* imaging to compare STM images with AFM images of an electrochemically controlled surface with molecules adsorbed on it.

We have begun to investigate chemical methods for tethering nucleic acids to graphite and gold substrates.

We have studied porphyrin molecules tethered to Au(111) by an isocyanate group. We have used model compounds (mono and di-porphyrins) to investigate the image contrast.

SIGNIFICANCE: We now understand that rather special conditions are required to react nucleic acids onto substrates in a manner that a) retains some semblance of their solution geometry and b) keeps them in place against the strong forces imposed by the STM and AFM tip as it scans. These conditions are difficult to duplicate when DNA is reacted onto a surface (the surface homogeneity suffers over the course of a reaction). Holding the molecules by polarization forces (*in situ imaging*) has proved to be spectacularly reproducible. Unfortunately, we have very little understanding of the factors that control the molecular conformation on the electrode (except that we know that it is altered strongly). Our studies of contrast formation indicate that, at the atomic level, STM images of biomolecules are almost impossible to interpret (we do not believe the published 'literal' interpretations) and even AFM images are not straightforward to interpret.

WORK PLAN (next 12 months): We are now convinced that we have a completely reliable method for imaging 'clean' biomolecules on atomically flat (and clean) gold. However, we cannot interpret the images: are the molecules denatured? Are they aggregated? Are they associated with features on the surface or with counter and co-ion

complexes? We believe that we can simplify the problem somewhat by using molecules that are engineered to take on a recognizable form and retain it in the presence of salt variations, etc. For this purpose, we have chosen small (radius ~ 100 Å) synthetic closed circles of double helical DNA. These are being synthesized and purified in collaboration with Rod Harrington's group at the University of Nevada, Reno. We are also planning more elaborate samples: e.g., with tethering sequences (for chemical tethering) at certain points on the circles, and with interesting sequences (e.g., *cro* binding site) at other locations. We are attempting a systematic study of the effects of counter- and co-ions and surface charge on the polymer geometry at the metal-liquid interface.

SELECTED PUBLICATIONS AND REPORTS (11 of 15):

1. "How does the Scanning Tunneling Microscope Image Biopolymers?" S.M. Lindsay, O.F. Sankey and K.E. Schmidt, Comments on Molecular and Cellular Biophysics, **A7**, 109-129 (1991).
2. "Electrical, Spectroscopic and Morphological Investigations of Chromium Diffusion through Thin Gold Films" M.A. George, W.S. Glaunsinger, T. Thundat and S.M. Lindsay, Thin Solid Films, **189**, 59-72 (1990).
3. "Modification of Tantalum Surfaces by Scanning Tunneling Microscopy in an Electrochemistry Cell" T. Thundat, L.A. Nagahara, S.M. Lindsay, M.A. George and W.S. Glaunsinger, J. Vac. Sci. Technol. **A8**, 3537-3541 (1990).
4. "Electrochemical Deposition of Molecular Adsorbates for In Situ Scanning Probe Microscopy" L.A. Nagahara, T. Thundat, P.I. Oden, S.M. Lindsay and R.L. Rill, Ultramicroscopy, **33**, 107-116 (1990).
5. "Nanolithography on Semiconductor Surfaces under an Etching Solution" L.A. Nagahara, T. Thundat and S.M. Lindsay, Applied Physics Letters, **57**, 270-272 (1990).
6. "Can the Scanning Tunneling Microscope Sequence DNA?" S.M. Lindsay and M. Philipp, Genetic Analysis **8**, 8-13 (1991).
7. "Scanning Tunneling Microscopy Investigations of Polysilicon Films under Solution" J.P. Carrejo, T. Thundat, L.A. Nagahara, S.M. Lindsay and A. Majumdar, J. Vac. Sci. Technol. **B9**, 955-959 (1991).
8. "A Technique for Stable Adhesion of DNA to a Modified Graphite Surface for Imaging by STM" Yu.L. Lyubchenko, S.M. Lindsay, J.A. Deroose and T. Thundat, J. Vac. Sci. Technol. **B9** 1288-1290 (1991).
9. "Electrochemical Deposition of Nucleic Acids for Scanning Probe Microscopy" J.A. Deroose, S.M. Lindsay, L.A. Nagahara, P.I. Oden and T. Thundat, J. Vac. Sci. Technol. **B9**, 1166-1170 (1991).
10. "Studies of the Electrical Properties of Large Molecular Adsorbates" S.M. Lindsay, Y. Li, J. Pan, T. Thundat, L.A. Nagahara, P.I. Oden, J.A. Deroose and U. Knipping, J. Vac. Sci. Technol. **B9** 1096-1101 (1991).
11. "Imaging Nucleic Acids with Scanning Probe Microscopes" S.M. Lindsay in *Biotech 1990*, (CMC, Washington) pp 62-67. (1990).

## ANNUAL PROGRESS REPORT

GRANT #: N00014-88-K-0322

R&T CODE: 4415805

PRINCIPAL INVESTIGATOR: Shalom R. Rackovsky

INSTITUTION: University of Rochester

GRANT TITLE: Quantitative Classification of Known Protein Structures.

REPORTING PERIOD: 1 July 1990-30 June 1991

AWARD PERIOD: 1 July 1988-30 June 1991

OBJECTIVE: To develop mathematical methods for comparing and classifying the structures of proteins of arbitrary molecular weight; to develop a data base of protein x-ray structures representative of the entire set of known protein structures; to apply the methods developed to the data base, in order to understand the structural relationships between the known protein structures; to develop new insight into aspects of protein folding and evolutionary relationships based on the results of the comparison and classification studies.

ACCOMPLISHMENTS (last 12 months): During this year, emphasis was placed on the generalization of the mathematical methods developed over the last two years to the classification of protein sequences. The 20 amino acids can be characterized in terms of their physical properties, using an exhaustive factor analysis of the available data carried out by the Scheraga group. Using these data, the sequences of proteins have been represented as a distribution of vectors, analogous to the Generalized Bond Matrix representation of structures developed over the last two years. This has made it possible to study the distributions of protein sequences in a high-dimensional sequence space, just as the distribution of structures was studied in a high-dimensional structure space. Methods are being developed for optimizing the correspondence between the two spaces, and the parameters relevant to the optimization explored. One ultimate goal is to use this correspondence to predict structural characteristics of proteins for which only a sequence is available. We would also like to determine which physical characteristics of the amino acids are responsible for various aspects of protein folding, and to shed some light on the types of pro-

tein sequence which exist.

We have also begun to study the relationship between sequence and structure using a new set of tools, adapted from mathematical cryptography. There is currently a large amount of discussion about the existence of a "second genetic code" which governs protein folding. It is hoped, by taking this metaphor seriously, to extract useful information on folding using cryptographic methods and the structural and sequence data which we have developed over the last two years.

Study continues of the role of length scale in the classification of protein structures. We are also continuing the development of tools for practical structural and sequence analysis based on the methods which we have developed. The manuscript on this topic which had been planned for this year has been deferred pending further work.

SIGNIFICANCE: The application of the methods we have developed to the study of sequences initiates a new phase of this work. It is hoped that, by studying structures and sequences in parallel, we will be able to gain new insight into the relationship between the two. Exploitation of the analogy between protein folding and cryptography is expected to make available a whole new approach to the field.

Work Plan (next 12 months): It is planned to continue the work on sequence classification and sequence structure correlation, with a view to ascertaining whether there exist well-defined sequence types, and how good a correlation exists between the protein sequence and structure spaces. Intensive study of the cryptographic aspects of protein folding is planned, and further development of the application tools will take place.

Publications and Reports (last 12 months):

Quantitative Organization of the Known Protein X-Ray Structures. I. Methods and Short-Length-Scale Results. S. Rackovsky, PROTEINS: Structure, Function and Genetics 7, 378 (1990).

## ANNUAL PROGRESS REPORT

GRANT #: N00014-WR-24020

R&T CODE: 4415806

PRINCIPAL INVESTIGATOR: Lou Reinisch

INSTITUTION: Laser Biophysics Center Uniformed Services  
University of the Health Sciences

GRANT TITLE: Protein Dynamics Studied With Resonantly  
Enhanced Quasi-Elastic Light Scattering

REPORTING PERIOD: 1 July 1990 - 31 May 1991

AWARD PERIOD: 1 July 1988 - 30 June 1991

OBJECTIVE: We intend to measure protein fluctuation spectra using quasi-elastic light scattering. In many protein systems, the fluctuations exert partial if not a major control over the function of a protein. A complete understanding of the protein function and the control of protein function is not possible without understanding the protein fluctuations and how these fluctuations change with the protein environment.

ACCOMPLISHMENTS (last 11 months): We have observed the transient changes in the curvature of purple membrane fragments upon photoexcitation as a function of the pH of the suspending medium. The room temperature suspensions have low ionic strengths, and the bending is observed by changes in the scattered light at 320 nm. We have developed a first order approximation to subtract away any changes made during the photocycle, and we have validated this first order approximation. The resultant scattering curves as a function of pH have been fit to the sum of three fundamental bending modes. We have mathematically defined a fundamental bending mode with the following equation:

$$\Delta I/I = F[\exp(-t/\tau_d) - \exp(-t/\tau_r)]$$

where I is the intensity of the scattered light,  $\Delta I$  is the transient change in the scattering, F is the amplitude of the fundamental bending mode, and  $\tau_r$  and  $\tau_d$  are the rise and decay of the bending process, respectively. We have modelled two of the processes as transient forces correlated with the charge motion during the photocycle. The third process is probably caused by local changes in the pH as the protein pumps protons, or changes in the surface charge density as nonproton ions are released during the photocycle. If suspension is excited with UV light (355 nm), the first two processes related to the charge motion during the photocycle are present. However, the third process is missing. This result is important for two reasons. First, our deconvolution of the scattering transients into three fundamental bending modes was

originally a mathematical fit to the data. Since one mode is missing with the UV excitation, the three fundamental bending mode model is valid. Second, the transient absorption changes during the proton pumping process of bacteriorhodopsin shows no difference between the visible and UV excitation. However, our probe of membrane motion is more sensitive to changes in the protein structure and shows a difference between the visible and UV excitation.

SIGNIFICANCE: The membrane bending is significant in the study of bacteriorhodopsin. Measurements of the bending as a function of the pH has revealed an unexpected richness in the data. Two of the fundamental bending processes have been associated with the protein conformational changes during the proton pumping process. One of the fundamental bending processes has been circumstantially linked to a change in the ion concentration in the vicinity of the membrane. Also, the transient, scattering amplitude is very sensitive to low levels of photodestruction. Thus, the transient scattering is sensitive to critical aspects of bacteriorhodopsin, where the absorbance measurements do not show such sensitivity. Understanding the changes in the scattering and electrostatic interactions in the protein is important in understanding the mechanism of the proton pump and the mechanism of the proteins.

WORK PLANS (next 12 months): The grant ends. Computer calculations to support our model will continue.

PUBLICATIONS AND REPORTS (last 12 months):

1. J. Czege and L. Reinisch, Photo-destruction of Bacteriorhodopsin, Photochem. Photobiol. 53:659-666 (1991).
2. J. Czege and L. Reinisch, The pH Dependence of Transient Changes in the Curvature of the Purple Membrane, Photochem. Photobiol. 00:0000-0000 (1991).
3. J. Czege and L. Reinisch, The pH Dependence of Transient Changes in the Purple Membrane with Visible and Ultraviolet Excitation. Biophys. J. (submitted).
4. J. Czege and L. Reinisch, The Transient Changes in the curvature of the Purple Membrane Fragments as a Function of pH Using Visible and Ultraviolet Excitation, Biophysical J. 59:381a (1991).
5. J. Czege and L. Reinisch, Huge Components in the Scattering Kinetics During the Photocycle of Triton Treated Purple Membrane, Am. Assoc. Adv. Science Annual Meeting, 148 (1991).
6. J. Czege and L. Reinisch, Symmetry Violation in the Proton Pump of Bacteriorhodopsin, Bull. Am. Phys. Soc 36:549 (1991).

## ANNUAL PROGRESS REPORT

GRANT #: N00014-SJ-C-0293

R&T CODE: 441p019

PRINCIPAL INVESTIGATOR: Anthony J. Salerno

CORPORATION: Allied-Signal Inc.

GRANT TITLE: Synthesis and Characterization of High Molecular Weight Adhesive Polypeptides Containing L-DOPA Residues

PERIOD OF PERFORMANCE: 1 June, 1990 - 1 June, 1991

AWARD PERIOD: 1 October, 1989 - 30 September, 1991

OBJECTIVE: To develop a generic technology for the large-scale biological production of repetitive proteins using the consensus decapeptide of *M. edulis* as a model.

ACCOMPLISHMENTS: Experiments were completed that were designed to address the nature of the remarkable differential production of polydecapeptide precursor in T7-based vs.  $\lambda p_L$  promoter-based *E. coli* expression systems. An isogenic panel of hosts was constructed that allowed analysis of the components of the two systems. The T7 induction system per se (promoter and controlling means) is largely responsible for the impressive yields of polydecapeptide precursor attainable.

Production of polydecapeptide precursor has been examined in bioreactor studies ranging in size from 1.5 to 5.0 liters. These studies are ongoing.

Characterization of stability of repeating DNA entailed several experiments based on two major experimental approaches. The first involved the development of a general quantitative assay for determining deletion rate that would be expressed as the percent of a gene cassette population undergoing deletion per plasmid replication. The utility of such an approach is two-fold. First, it provides a means to predict the stability at a given scale of fermentation early in a project. Secondly, if adopted by the scientific community working in this field, it provides an objective comparison of the relative stability of the various repetitive genes under investigation and development. The approach relies on the ability to accurately determine the number of cell generations, the plasmid copy number, and the percent of gene cassette which is partially deleted. Implementation of this approach has been hampered by a number of problems, the most serious of which is a stochastic or physiological selective replication of plasmids carrying partially deleted gene cassettes. When this occurs during

the first few plasmid generations, a severe distortion occurs in the population of deleted plasmids which results in "erroneously" high deletion rates. Thus the technique is reduced to one giving a "semi-quantitative estimate" of deletion rate. Pilot experiments were performed to see how the estimated deletion rate obtained with this technique correlated to earlier observations on the effect of copy number, gene length, repeat length, and sequence. In general, the data corresponded well to earlier observations on these variables.

The second approach involved construction of a repeating-gene stability probe vector to use as a screening tool to isolate strains better able to propagate repetitive DNA. This stability-probe vector, pSP1, is based on a replicon (pOU71) temperature-sensitive for replication. Below 37°C the plasmid is present at one copy per chromosome, whereas at 42°C the copy number increases to more than 1000 plasmid molecules per cell. pSP1 contains two collagen-analog genes (cags) in tandem, each approximately 400 bp long. Inserted between these two genes is a kanamycin-resistance ( $Km^R$ ) determinant located on a 1.1 kb DNA fragment. Cultures were grown in a *recA*<sup>-</sup> host at 30°C, 37°C, or 42°C for approximately 70 generations and then screened for loss of  $Km^R$ . Results showed that the  $Km^R$  determinant was lost very infrequently (<0.3%). Southern blot analysis confirmed that, as expected, all  $Km^S$  colonies contained only a single cag. However, analysis of some randomly picked  $Km^R$  colonies also showed in each case significant deletions in the cags as well as bands larger in size than the original cags. Deletions across the  $Km^R$  determinant occurred only rarely in *recA*<sup>-</sup> and *recA*<sup>+</sup> strains.

**SIGNIFICANCE:** The results on the relative effectiveness of the T7 expression system reinforce the notion that it is the system of choice for the intracellular production of polydecapeptide-analog precursor in *E. coli*.

Multiple variables impact repetitive gene stability in production strains. In essence, cellular growth results in a population of cells containing a population of plasmid carrying a population of partially deleted repeating gene cassette. Environmental, physiological, and/or stochastic selection of elements within each statistical population act in concert to make the accurate, quantitative determination of deletion rate in the actual production strains a daunting task.

Studies with the stability probe vector show that deletion events in short tandem repeat DNA are not *recA* dependent and do not seem to occur primarily by homologous recombination.



ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-3078

R&T CODE:441p029

PRINCIPAL INVESTIGATOR: Nadrian C. Seeman

INSTITUTION: New York University

GRANT TITLE: Macromolecular Design: Structural Engineering of  
New Biomaterials on the Nanometer Scale

PERIOD OF PERFORMANCE: 1 June, 1990--31 May, 1991

OBJECTIVE: To explore the feasibility of using branched nucleic acid molecules as a basis for constructing N-connected objects on the nanometer scale.

ACCOMPLISHMENTS (last 12 months): We have completed our isolation and preliminary characterization of a DNA cube. This cube consists of 6 cyclic single strands of DNA, each corresponding to a face of the molecule. In order to complete our proof of synthesis we had to separate the 6-cycle target molecule from the 5-cycle failure product. This is not possible on a routine 5% bisacrylamide denaturing gel, but we were able to achieve the necessary separation by using a 1.25% bisacrylamide gel (13% total polyacrylamide).

We have analyzed the mobility of the cube and its 5-cycle, two 4-cycle and two 3-cycle sub-structure catenanes as a function of acrylamide and bisacrylamide concentration in both native and denaturing gels. We are able to separate all these species, including the two 4-cycle and two 3-cycle topoisomers. The only constant correlation found involves the apparent time-averaged symmetry of the structure--the higher the symmetry, the lower the mobility.

Although we are able to control the connectivity of the cube, we are unable to control the direction of cyclization in the last step. Thus, we do not know whether the belt closed in front of, or behind the page. We have tested base I sensitivity on squares, as a handle on this problem.

The number of structures that can be made from 3-arm junctions and 4-arm junctions is limited. We have, for the first time, synthesized 5-arm and 6-arm junctions, and we have characterized these molecules. The 5-arm junctions have no dominant stacking domains, but the 6-arm junction has a single dominant stacking domain.

The difficulties encountered in the synthesis of the cube have spurred us to develop a more general and potentially automatable procedure for the assembly of DNA objects. Accordingly, we have emulated the procedures used to synthesize linear DNA chemically: We have developed a solid-support procedure for the DNA object assembly. The highlight page illustrates the use of the solid support procedure to synthesize a square, which we have done as a test. A tether is synthesized on the support (black ball in the diagram), and a crosslinked duplex is ligated to it (indicated by horizontal stripes). As shown, a closed junction is added to the support, and then it is restricted to 'deprotect' an arm. A newly closed junction is then added, and it is restricted, in turn. The restriction sites are destroyed each time, because they are distal or interrupted sites. On the final step, two restrictions are performed, and the structure is closed. Although a junction is shown as being added, the added material can, in principle, be a DNA polygon or a DNA polyhedron. Purification

is achieved on the support by exonuclease treatment of failure products. Although a single enzyme is shown as being used, we have found that a cycle of restriction enzymes is more useful, in that an unrestricted site (failure to deprotect) can be rescued by restriction on a second pass.

**SIGNIFICANCE:** We have completed the synthesis and preliminary characterization of the first closed N-connected object ( $N > 2$ ) to be constructed from DNA. As the cube-like object is a very bad approximation to the sphere, with a lot of phosphate-phosphate repulsion to be expected, we expect that less crowded polyhedra can be fabricated with greater ease.

The development of 5-arm and 6-arm junctions will permit the synthesis of 5-connected and 6-connected objects, as well as less strained 4- and 5- connected objects, if an external arm is left relaxed. The synthesis of icosahedral deltahedra (dual structures to 'Bucky Balls') which form the frameworks of geodesic domes and many viruses can now be contemplated with this system. In addition, 11 new 432-symmetric lattices are also possibly feasible now.

The solid-support synthetic procedure will permit the construction and purification of complex objects with this system. We expect that the ability to purify materials on the support by destroying failure molecules (analogous to 'capping' in DNA synthesis) will greatly improve the ability to isolate target molecules.

**WORK PLAN (next 12 months):** Our main goals for the coming period involve refinement of the solid-support synthetic methodology, and its utilization in the synthesis of a larger polyhedron. Currently we are targeting a truncated octahedron, to be assembled from DNA squares. We expect to get a good start on this during the coming year.

The characterization of the inside and outside of the DNA cube should be a reachable goal during the coming year as well. If the DNase I susceptibility does not work, addition of proteins to particular residues may be used as an alternative strategy.

The 5-arm and 6-arm junctions reported have 16 nucleotide pairs per edge, rather than 8, as is possible with 3-arm and 4-arm junctions. We are trying to find the minimum size arms necessary for these junctions. We have already demonstrated that 12 nucleotide pairs can work, and will soon investigate the stability of 10 nucleotide pairs.

#### PUBLICATIONS AND REPORTS:

N.C. Seeman, *De Novo Design of Sequences for Nucleic Acid Structure Engineering*, *Journal of Biomolecular Structure and Dynamics* **8**, 573-581 (1990).

J. Chen and N.C. Seeman, *The Synthesis from DNA of a Molecule with the Connectivity of a Cube*, *Nature* **350**, 631-633 (1991).

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1529

R&T CODE: 441p024

PRINCIPAL INVESTIGATOR: Anthony J. Sinskey

INSTITUTION: Massachusetts Institute of Technology

GRANT TITLE: Strategies for Biopolymer Engineering of Polyhydroxy-Butyrate-Like Materials

REPORTING PERIOD: 1 April 1991 - 1 June 1991

AWARD PERIOD: 1 April 1991 - 14 March 1993

OBJECTIVE: The overall objective of this project is to study the genetics and enzymology of polyhydroxyalkanoate (PHA) metabolism at the molecular level in order to understand how this process functions. From this information we will then be able to engineer new production strains, utilizing bacterial and plant systems, in which the composition and molecular weight of the PHA biopolymers can be controlled.

### ACCOMPLISHMENTS:

#### PHB Biosynthesis

In the past few years efforts have been successful in characterizing the genes and gene products responsible for the biosynthesis of PHB, from two organisms, Zoogloea ramigera and Alcaligenes eutrophus. Mutagenesis studies of the active site base, Cys378, of the  $\beta$ -ketothiolase enzyme have now essentially been completed. Unlike the inactive Gly378 mutant, the Ser378 enzyme retains some activity although the  $K_{cat}$  in the overall forward and reverse reactions is 1000-fold lower than the wild type. In addition, partitioning of the acyl-enzyme intermediate in the first half reaction has been reversed (manuscript in preparation).

A series of experiments designed to overproduce the PHB polymerase enzyme in E. coli using different promoter/vector systems have proved unsuccessful so far. The main problem being that if you provide a ribosome binding site/ N-terminal coding sequence which will be efficiently translated then the protein forms inclusion bodies and is inactive (we have encountered the same problem with the P. oleovorans PHA polymerases). However, the inclusion bodies do account for as much as 20-30% of the total cell protein and can be purified and solubilized. We have also started to engineer E. coli strains in which the thiolase-reductase enzymes are expressed at different levels from the A. eutrophus phbAB genes integrated into the chromosome. These strains will be used to overproduce the PHB polymerase. In the absence of an overproduction/purification system for the polymerase, we have initiated mutagenesis studies on the enzyme and are testing the mutants for complementation of PHB polymerase negative mutants.

The expression of the phbCAB genes have been studied using  $\beta$ -galactosidase fusions and two promoters identified and mapped (Peoples, O.P. and Sinskey, A.J. manuscript in preparation).

#### Pseudomonas oleovorans TF41L PHA Locus

Previously we reported the isolation of the PHA polymerase locus from P. oleovorans and identified three open reading frames. The predicted polypeptide products of ORF1 and ORF3 having 40% and 42% absolute identity with the A. eutrophus PHB polymerase enzyme (Peoples and Sinskey, 1990).

We have now completed the characterization of this locus in more detail (Peoples, O.P., Lim, P.-H. and Sinskey, A.J. manuscript in preparation). ORF1 complements all of the PHA-negative strains we have analyzed and encodes the PHA polymerase. Overexpression of this gene in

the wild type strain results in a two-fold increase in PHA production. Overproduction of ORF2, essentially eliminates the accumulation of PHA, supporting the identification of this gene product as the PHA depolymerase on the basis of complementation analysis (Witholt's group, Netherlands). The role of ORF3 in PHA metabolism remains unclear, although it is probably a PHA polymerase gene. It is worth noting the two large inverted repeat structures, the first (IR1, nucleotides 3163-3228) located between ORF2 and ORF3 and the second (IR2, nucleotides 5603-5583) located downstream of ORF3. The stem regions of these two structures are almost identical in sequence except for two nucleotides (nucleotides 3217-3218 of IR1) which in the case of IR1 represents a potential translation initiation codon.

Analysis of the expression of each of the reading frames using  $\beta$ -galactosidase gene fusions indicates that all three reading frames are expressed from a single promoter located upstream of ORF1. This promoter has been identified by S1-mapping and growth studies indicate that it is transcriptionally regulated although the mechanism remains to be determined.

#### WORK PLAN:

In the next year, we will focus primarily on overproduction/purification of the PHB polymerase and will use several new E. coli strains in which we can control the availability of D- $\beta$ -hydroxybutyryl-CoA through manipulation of the phbAB genes. By providing the correct environment we should be able to produce correctly the folded polymerase at high levels bound to PHB oligomers.

Mutagenesis studies on the PHB polymerase will continue to be performed using complementation and production of PHB in E. coli as tests for activity. In particular we will finish the experiments to replace Cys319 and Cys459, both of which are conserved in the P. oleovorans PHA polymerases. Deletion analysis to identify regions of this protein essential for binding to the PHB granules will be carried out.

Transcriptional regulation of the P. oleovorans pha locus has been demonstrated. One of the questions we will address is the identification of the pha locus promoter at which this regulation is effected.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-88-K-0323

R&T CODE: 441n009

PRINCIPAL INVESTIGATOR: James M. Stewart

INSTITUTION: University of Maryland - Code 07419

GRANT TITLE: Macromolecular Calculations for the Xtal  
System of Crystallographic Computer Programs

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 June 1988 - 31 May 1991

OBJECTIVE: To produce, within the Xtal system of crystallographic computer programs, codes, documentation and tests from macromolecular crystallographic calculations.

This project is being carried out in collaboration with K. B. Ward and D.M. Collins of the Naval Research Laboratory (Code 6030).

ACCOMPLISHMENTS: During the past year, the major effort has been on the coding and preliminary testing of the program MEMPHAS. This program presents the exciting possibility to determine protein structures directly from the diffraction data of the native protein substance. The program MEMPHAS uses a maximum entropy algorithm for the ab-initio determination of crystallographic phases. This algorithm is based on the work of D. Collins at the Naval Research Laboratory and E. Prince at the National Institute for Science and Technology. MEMPHAS is based on programs written at NIST by E. Prince and translated into the Xtal system and utilizing features coded into Xtal under earlier years of this support. This program shows great promise and should, if the method proves applicable, have a profound impact on protein crystal structure analysis. As this support draws to a close the programming is far advanced, the documentation has been partially prepared, and preliminary testing has been done. The results of these tests are, so far, very encouraging. Work will continue in an effort to establish a strategy for using this tool in structure determination.

In addition to the MEMPHAS program, a previously developed code, MIR, which produces phases for the native protein from multiple isomorphous replacement data has revision and checking. This large program is the mainstay of the conventional method of protein crystal structure solution and a very important link in the Xtal system.

During this final year a great deal of effort went into the implementation and checking of the whole system in its

latest release (Xtal3.0) on three machines at the Naval Research Laboratory and one at the University of Maryland. The Cray, VAX, Silicon Graphics and UNISYS machines have been used to establish that the whole system is interchangeable among these four machines with their disparate operating systems.

#### RECAPITULATION OF PRIOR PROGRESS MADE

During the course of support of this project a number of important macromolecular programs have been included in the Xtal system. These programs now exist in version 3.0 which was distributed from Prof. Sydney Hall from the university of Western Australia at the end of 1990. Documentation which was generated under this support is now included in the Xtal version 3.0 manual which was distributed in November 1990.

This edition of the Xtal system is being distributed through Dr. S.R. Hall, Crystallography Centre, University of Western Australia, Nedlands, 6009.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1970

R&T CODE: 441p016

PRINCIPAL INVESTIGATOR: Dan W. Urry

INSTITUTION: University of Alabama, Birmingham

GRANT TITLE: Development of Elastomeric Polypeptide Biomaterials

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 June 1990 - 31 May 1992

### OBJECTIVE:

Several important objectives of this research effort have been met in the past year: 1. to determine the effect on the temperature of the inverse temperature transition,  $T_i$ , of each of the naturally amino acid residues as a guest residue, X, in poly[ $f_v(\text{VPGVG})$ ,  $f_x(\text{VPGXG})$ ] where several  $f_v$  and  $f_x$  are mole fractions, 2. to determine the effect of pressure on  $T_i$  for several guest residues, and 3. to express sufficient (VPGVG)<sub>n</sub> from a microbial source to allow for physical characterization. Objective 1. is necessary for the general development of elastomeric polypeptide biomaterials, whereas objectives 2. and 3. are more specific developments.

### ACCOMPLISHMENTS (last 12 months):

While there are yet values to improve, the temperature of the inverse temperature transition,  $T_i$ , has been evaluated for each of the amino acid residues, including, where relevant, their different states of ionization. With the general formula poly-[ $f_v(\text{VPGVG})$ ,  $f_x(\text{VPGXG})$ ], values of  $T_i$  for  $f_x = 1$  directly determined or extrapolated are given in Figure 1 which includes the plots of  $f_x$  vs  $T_i$ . With the appropriate mixing of amino acids for specific desired properties of pH, salt, and pressure sensitivities, polymers can be designed with the capacity to set  $T_i$  where desired. As will be seen below, what has been obtained is a particularly functional hydrophobicity scale in general for proteins.

It has been demonstrated that the effect of pressure on  $T_i$  is small for residues such as Met, Val, Pro and Gly, but it is more than an order of magnitude greater for the aromatic residues, Trp, Phe and Tyr, with the calculated values for  $\Delta V$  being more than two orders of magnitude greater for Trp and Phe than for Val. This provides the information to design polypeptides capable of baromechanical and barochemical transduction.

The third major accomplishment achieved in the past year involved the molecular biology component of the project. Synthetic oligonucleotides had been constructed with a sequence coding for (VPGVG)<sub>10</sub> which through the use of PCR and subsequent cloning steps resulted in a gene that expresses G-(VPGVG)<sub>10</sub>-VPGV as a C-terminal fusion to glutathione S-transferase (gst) from the pGEX-3X vector. The fusion protein, gst-G-(VPGVG)<sub>10</sub>-VPGV, contains the recognition sequence for protease factor Xa at the fusion junction. The fusion gene was expressed to high levels in E. coli purified and cleaved to produce 15mg quantities of G-(VPGVG)<sub>10</sub>-VPGV from a single twelve liter fermentation. The product was characterized by proton and carbon-13 NMR and the value of  $T_i$  was determined for a 40 mg/ml solution.

### SIGNIFICANCE:

Because the temperature is maintained constant in warm-blooded (homiothermic) animals, whether or not a given polypeptide or protein is hydrophobically folded does not depend on the endothermic heat of the

inverse temperature transition; it depends only on whether the temperature of the inverse temperature transition is above or below the physiological temperature. Thus at 37 °C poly(VPGVG), poly(VPGLG), poly(VPGMG), and poly(VPGIG) are folded, whereas poly(VPGSG), poly(VPGSG), poly(VPGAG) and poly(VPGNG) are not folded. The hydrophobicity scale (Figure 1) can be used directly to identify transmembrane helices in bacteriorhodopsin, halorhodopsin, glycophorin, and human cytochrome b<sub>5</sub> (Figure 2). A sequence with a mean transition temperature,  $\langle T_i \rangle_{11}$ , of less than 37 °C should be hydrophobically folded or interacted.

The central issue of protein folding by means of hydrophobic interactions now focuses on the means whereby the temperature of the folding transition can be changed. As shown in Figure 1, the change in the ionized state has a remarkable effect on the value of  $T_i$ ; the most dramatic effect, however, is that of phosphorylation/dephosphorylation. It is now quite apparent why phosphorylation/dephosphorylation is a dominant means whereby protein structure and function are achieved. Also the non-covalent binding of chemical moieties to the polypeptide or protein can change the value of  $T_i$  as can changes in solvent composition (2,6) and pressure (1). Thus a unifying mechanism, that of controlling the temperature of the inverse temperature transition of protein folding and assembly, is emerging for understanding free energy transduction in protein.

The observation of the pressure effect on changing  $T_i$  for aromatic residues will allow for the design of matrices which will exhibit baromechanical and barochemical transduction.

The production of significant amounts of poly(VPGVG) using the pGEX-3X vector will allow for the design of sequential polypeptides that are presently beyond the capacity of chemical syntheses and hopefully will lead to the most inexpensive production of elastomeric polypeptides biomaterials.

#### WORK PLAN (next 12 months):

The planned research for the coming year continues as originally proposed with the specific elements (1) of carrying out the differential scanning calorimetry for the entire set of some fifty polymers of the general formula,  $\text{poly}[f_v(\text{VPGVG}), f_x(\text{VPGXG})]$  where x is each of the naturally amino acids in each of their accessible states, (2) of constructing a pressurizable cell in order to demonstrate baromechanical and barochemical transduction, and (3) of constructing sequential polypeptides by means of recombinant DNA methodology, specifically to achieve higher molecular weights of poly(VPGVG) and to insert additional repeating sequences such as  $[(\text{VPGVG})_1-(\text{VAPGVG})_m]_n$ .

#### SELECTED PUBLICATIONS AND REPORTS (3 of 13):

1. Dan W. Urry, Larry C. Hayes, D. Channe Gowda, and Timothy M. Parker, "Pressure Effect on Inverse Temperature Transitions: Biological Implications," In Chemical Physical Letters (A.D. Buckingham, Ed.) Elsevier Science Publishers (North-Holland Physics Publishing Div.), Amsterdam, The Netherlands (in press).
2. Chi-Hao Luan, and Dan W. Urry, "Solvent Deuteration Enhancement of Hydrophobicity: DSC Study of the Inverse Temperature Transition of Elastin-based Polypeptides," Journal of Physical Chemistry, 1991 (in press).
3. Chi-Hao Luan, and Dan W. Urry, "Molecular Mechanics Study of the  $\beta$ -spiral Conformations of the Phe<sup>4</sup>, Tyr<sup>4</sup>, and Trp<sup>4</sup> Analogs of the Elastomeric Poly(Val<sup>1</sup>-Pro<sup>2</sup>-Gly<sup>3</sup>-Val<sup>4</sup>-Gly<sup>5</sup>)," (Int'l J. of Quantum Chem. Paper Symp.-Clementi-Volume), March 1991.



## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-3121

R&T CODE: 441030

PRINCIPAL INVESTIGATOR: J. Herbert Waite

INSTITUTE: University of Delaware/ College of Marine Studies

GRANT TITLE: Marine Cement: Anatomy of a Natural Composite Material

REPORTING PERIOD: 1 Nov 1990 - 31 Oct 1992

AWARD PERIOD: 1 Nov 1989 - 31 Oct 1992

OBJECTIVES: To characterize the proteins involved in the formation of the mussel byssus and to explore the nature of the molecular interactions between these proteins.

ACCOMPLISHMENTS (last 12 months): Of the four known proteins in byssus, i.e. polyphenolic (glue) protein, collagen, catecholoxidase and varnish protein, progress in the characterization of two has recently occurred. Varnish protein is acid-extracted from the accessory gland, and although it contains Dopa like the adhesive polyphenolic protein, the levels are much lower. The other differences include a higher content of Asx, Gly and Pro. There is no hydroxyproline. In contrast to the polyphenolic protein, the varnish protein is exceptionally resistant to proteases (V-8, trypsin, chymotrypsin, pepsin) and even partial proteolysis by trypsin requires 48 h at enzyme/protein ratios of 1:1. The N-terminus shown in Fig. 1 is distinct from that of the polyphenolic protein. Tryptic peptide T-7 is also distinct although some similarities (underlined portion) such as the tandem prolines and Dopa persist, and might be used to explain the immunologic cross-reactivity of plaque and varnish observed by Benedict and Waite (1986). The varnish protein precursor has an apparent molecular weight of 45 kDa and pI of 8.5. Monospecific polyclonal antibodies to the varnish protein are in preparation.

Collagen, the load-bearing fibre in byssus, is not directly extractable from byssus. A large (55 kDa) fragment, however, is following extensive proteolysis under acid conditions with pepsin. The fragment is purified by reversed phase HPLC and has both a composition and sequence that is typical of other interstitial collagens with gly at every third position such as Gly-X-Y and pro/ Hyp occupying position Y (Fig. 1).

Catecholoxidase, which is virtually impossible to isolate in active form from the byssus, has been cloned in E. coli  $\lambda$ gt11. About  $10^5$  have been screened with a polyclonal antibody. Of these 17 are positives including 14 that contain an insert of about 1400 base pairs. Immediate plans include of the Eco RI insert into an M13 vector for sequencing.

SIGNIFICANCE: Byssal threads are coated over their entire surface by a protective varnish. The varnish is intriguing not only because of its clean glossy finish, but also because it effectively coats an apparently "pedestrian" collagen core from maine microbial attack. The chemical and physical stability of the byssus in sea water makes the interactions between the various proteins of particular interest.

WORK PLAN (next 12 months): The final term of this grant will be devoted to the immunohistochemical (colloidal gold) localization of the varnish protein within the secretory granules of the accessory gland; sequencing of the cDNA corresponding to catecholoxidase.

PUBLICATIONS AND REPORTS (last 12 months):

1. Waite, J.H. (1990) Phylogeny and chemical diversity of quinone-tanned

glues and varnishes (invited review). *Comp. Biochem. Physiol. B*, 97, 19-29.

2. Rzepecki, L.M., Nagafuchi, T., and Waite, J.H. (1991)  $\alpha,\beta$ -DehydroDOPA derivatives: Potential intermediates in natural composite materials. *Arch. Biochem. Physiol.* 285, 17-26.

3. Rzepecki, L.M., and Waite, J.H., (1991)  $\alpha,\beta$ -DehydroDOPA derivatives: Rate and mechanism of formation. *Arch. Biochem. Physiol.* 285, 27-36.

4. Waite, J.H. (1990) Detection of peptidyl-DOPA by amino acid analysis and microsequencing techniques. *Anal. Biochem.* 192, 429-433.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1551

R&T CODE: 441pin2

PRINCIPAL INVESTIGATOR: Dr. Joseph A. N. Zasadzinski

INSTITUTE: University of California, Santa Barbara

GRANT TITLE: Atomic Force Microscopy of Langmuir-Blodgett Lipid and Lipid-Protein Monolayers and Bilayers

REPORT PERIOD: 1 July 1990 - 1 June 1991

AWARD PERIOD: 1 January 1990 - 31 December 1992

OBJECTIVES: 1) To examine the organization of hydrated single and mixed lipid Langmuir-Blodgett films in various stages of aggregation to characterize these materials as matrices for proteins. 2) To examine LB films as models of two-dimensional phases and as model biomembranes 3) To improve the processing of LB films by identifying characteristic defects in LB films at nanometer resolution and to perhaps use LB films as templates for nano-lithography. 4) To better understand the imaging process in the AFM by examining surfaces of varied surface charge density, lateral mobility, hydrophobicity, hydrophilicity, etc.

ACCOMPLISHMENTS (last 12 months): We have demonstrated that the AFM can resolve defects (pinholes, tears, etc.) in a cadmium arachidate monolayer. The smallest holes we observed, about 10nm wide, were at least an order of magnitude smaller than any that had been visualized before by any technique. As a result of our work, the AFM should become the technique of choice in examining LB film quality. We also showed that it is possible to cut patterns into a LB film with a line width approaching 10nm as a possible masking step for nanolithography.

Brenda Northern (a student in my laboratory) found that the appearance of the mica surface lattice can change dramatically upon treatment with dilute acids or ion solutions. After treatment with HCl, the Oxinium ion ( $\text{H}_3\text{O}^+$ ) is known to readily replace the loosely bound potassium ions that normally provide charge neutrality to the mica surface. We see that the normal hexagonal "beehive" appearance of the mica lattice is replaced by a hexagonal array of bright dots, which correspond to the oxinium ions filling holes left by the potassium ions.  $\text{Al}^{3+}$  ions also bind tightly to the mica, but do not necessarily bind at every lattice site due to their higher valence. The apparent height of the  $\text{Al}^{3+}$  ions is also greater, although we do not yet know if this is because of the greater size of the hydrated ion and its counterions, or if the greater charge induces a stronger interaction with the AFM tip.

In collaboration with Drs. Jordan and Wilson of the UCSB's Biology Department, we have imaged microtubules from bovine brain to compare them with TEM images, with the goal of resolving molecular structure. Our highest resolution image so far has been of microtubule associated proteins. We are attempting to visualize the process by which microtubules assemble, and how vinblastine, one of a number of antimitotic drugs used in cancer chemotherapy, inhibits microtubule assembly. As in many investigations with AFM, one of the biggest obstacles was to determine how to immobilize the microtubules. We have done so by applying glutaraldehyde-fixed microtubules to a glow-discharged glass slide. The glow discharge makes the glass surface extremely hydrophilic, which causes the microtubules to bind strongly.

Along a different line, we have also been investigating the properties of SP-B protein important to lung surfactancy. We have obtained purified peptide fractions and antibodies from our collaborators,

A. Waring and H.W. Tausch at Drew/King Medical School. Flow-visualization experiments (which involve gentle swelling and vesicle formation from phospholipids commonly used in replacement lung surfactant therapies) show that an amphiphilic peptide from one end of the SP-B protein binds to vesicles as evidenced from fluorescent antibody labeling. Interestingly, the peptide from the other end of the SP-B protein does not label with a fluorescent antibody. We believe this suggests that the SP-B protein is inserting into the bilayer and acts as a transmembrane protein, and that we can chemically identify the region of insertion, and the region exposed. As such, it should be an excellent candidate for visualization studies as it will be well anchored in the lipid matrix, we completely know the amino acid sequence, and we know which end is protruding from the membrane. This flow-visualization has been developed in our lab by a second graduate student partially supported on this contract, Ms. M. Longo.

WORK PLAN (next 12 months): Since we have discovered nanometer scale defects in our LB films, we have been working to improve our film making technique, and trying to optimize our LB trough. This involves improvements in cleaning, vibration isolation, and temperature control. To be confident that we are observing equilibrium structures with the AFM, we must be sure that defects and other non-equilibrium effects are minimized. We also plan to visualize self-assembled monolayers of alkylsiloxanes on silica as a substrate for bilayer deposition. We have entered into a collaboration with Dr. G. Whitesides group at Harvard to provide us with the best possible self-assembled films for imaging. We also plan to work on modifying our NANOSCOPE II AFM to provide us with additional information on the local friction on the surface. This will tell us how to correct our images for secondary effects of imperfect cantilevers. We plan much more extensive imaging on DMPE bilayers to establish the existence of the hexatic phase; after we accomplish this, we will systematically begin to study the phase diagram of DMPE to see if more fluid phases of Langmuir-Blodgett films can also be imaged. Other lipids including DMPC and DMPG have been imaged and we will compare these materials to our DMPE images to determine how headgroup composition affects long-range ordering. We also plan to incorporate the SP-B protein into L-B films of DPPC or DPPE to try to image this hypothesized transmembrane protein. This would be very exciting as 1) no protein has ever been imaged before, and 2) the location and action of the SP-B protein is unknown, although it is known to be essential to its function in vivo.

PUBLICATIONS AND REPORTS (last 12 months):

1. J.A.N. Zasadinski, C.A. Helm, A.L. Weisenhorn, S.A.C. Gould, and P.K. Hansma, Atomic force microscopy of hydrated phosphatidylethanolamine bilayers. *Biophysical Journal*, 59: 755-760 (1991); with cover illustration.
2. H.G. Hansma, S.A.C. Gould, P.K. Hansma, H.E. Gaub, M.L. Longo, and J.A.N. Zasadinski, "Imaging Nanometer Scale Defects in Langmuir-Blodgett Films with the AFM. *Langmuir*, to appear June 1991.
3. J.A.N. Zasadinski, J.T. Woodward, M.L. Longo, and B. Dixon-Northern, "Scanning Probe Microscopy of Surfactant Monolayers and Bilayers," ACS Symposium Series on Macromolecular Assemblies, P. Stroeve and A. Balazs, editors, in press.
4. J.A.N. Zasadinski, "AFM of Langmuir-Blodgett Monolayers and Bilayers," to appear in *Proc. of the 49th Annual Meeting of the Electron Microscopy Society of America*, August, 1991.
5. B.L. Dixon-Northern, Y.L. Chen, J.N. Israelchvili, and J.A.N. Zasadinski, "AFM of Mica Surfaces After Ion Replacement," to appear in *Proc. of the 49th Annual Meeting of the Electron Microscopy Society of America*, August, 1991.

## **BIOREMEDIATION**

### **CORE PROGRAM**

**SCIENTIFIC OFFICER: DR. ERIC EISENSTADT**

**PROGRAM OBJECTIVE: TO DEVELOP THE BASIC PRINCIPLES GOVERNING THE ESTABLISHMENT AND MAINTENANCE OF MICROBIAL CONSORTIA IN MARINE ENVIRONMENTS, THE REGULATION OF BIODEGRADATIVE ACTIVITIES IN SITU, AND ENZYMATIC MECHANISMS OF ANAEROBIC BIODEGRADATION.**

**NAVY OBJECTIVE: TO DEVELOP EFFICIENT AND AFFORDABLE TECHNOLOGIES FOR ELIMINATING HAZARDOUS SUBSTANCES IN NAVAL ENVIRONMENTS.**

ANNUAL PROGRESS REPORT

GRANT #: R&T CODE: 4412200

PRINCIPAL INVESTIGATOR: Judith Bender

INSTITUTION: Clark Atlanta University

GRANT TITLE: Production and Characterization of  
Biofloculents in Metal-Sequestering  
Cyanobacteria Mats

REPORTING PERIOD: 1 April 1991 - 1 July 1991

AWARD PERIOD: 1 April 1991 - 31 March 1994

OBJECTIVE: Extracellular metal binding materials produced by microbial mats will be purified and characterized. The rate of metal removal will be determined. Genetic determinants for the major metal binding proteins will be identified. Protection of the microbes by extracellular material from the toxic effects of metals will be evaluated.

ACCOMPLISHMENTS: This is a new grant.

ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 4412201

PRINCIPAL INVESTIGATOR: Joseph J. Cooney

INSTITUTION: University of Massachusetts

GRANT TITLE: Marine Fungi as Novel Catalysts for  
Bioremediation of Oil Spills

REPORTING PERIOD: 1 April 1991 - 1 July 1991

AWARD PERIOD: 1 April 1991 - 31 March 1994

OBJECTIVE: To evaluate the ability of naturally occurring  
marine fungi to degrade hydrocarbons in oil.

ACCOMPLISHMENTS: This is a new grant.

ANNUAL PROGRESS REPORT

GRANT #: R&T CODE: 4412202

PRINCIPAL INVESTIGATOR: James G. Ferry

INSTITUTION: Virginia Polytechnic Institute and State University

GRANT TITLE: Anaerobic Degradation of Chlorinated Hydrocarbons by Marine Methanogens

REPORTING PERIOD: 1 April 1991 - 1 July 1991

AWARD PERIOD: 1 April 1991 - 31 March 1994

OBJECTIVE: Determine the enzymatic potential for dechlorination of chlorinated compounds in marine methanogenic environments. Characterize the enzymes with respect to electron donors, products, and reaction kinetics. Clone the genes encoding the responsible enzymes. Determine the potential of whole cells of marine methanogens to dechlorinate simple and complex compounds.

ACCOMPLISHMENTS: This is a new grant.



ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 4412203

PRINCIPAL INVESTIGATOR: Steve Goodwin

INSTITUTION: University of Massachusetts

GRANT TITLE: Pathway of Anaerobic Aromatic Hydrocarbon  
Degradation Coupled to Iron Reduction

REPORTING PERIOD: 1 April 1991 - 1 July 1991

AWARD PERIOD: 1 April 1991 - 31 March 1994

OBJECTIVE: Determine the metabolic pathway for the  
degradation of toluene by the anaerobic iron reducing  
bacterium, GS-15.

ACCOMPLISHMENTS: This is a new grant.

ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 4412204

PRINCIPAL INVESTIGATOR: Russell P. Herwig

INSTITUTION: University of Washington

GRANT TITLE: Biodegradation of Polychlorinated Biphenyls  
and Polycyclic Aromatic Hydrocarbons in  
Contaminated Marine Sediments

REPORTING PERIOD: 1 April 1991 - 1 July 1991

AWARD PERIOD: 1 April 1991 - 31 March 1994

OBJECTIVE: Determine the ability of naturally occurring organisms in marine sediment to metabolize hydrocarbons and PCBs. Characterize the microbial species in marine sediment consortia that metabolize these compounds. Develop bioreactors based on the ability of organisms from marine sediment to degrade hydrocarbons and PCBs.

ACCOMPLISHMENTS: This is a new grant.

ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 4412205

PRINCIPAL INVESTIGATOR: Holger W. Jannasch

INSTITUTION: Woods Hole Oceanographic Institute

GRANT TITLE: Bioremediation Studies with Bacterial Isolates  
from Extreme Marine Environments

REPORTING PERIOD: 1 April 1991 - 1 July 1991

AWARD PERIOD: 1 April 1991 - 31 March 1994

OBJECTIVE: Characterize the biodegradation activities of  
bacteria isolated from deep sea sediments containing high  
natural levels of petroleum.

ACCOMPLISHMENTS: This is a new grant.

ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 4412206

PRINCIPAL INVESTIGATOR: Joanne M. Jones

INSTITUTION: Naval Surface Warfare Center Detachment

GRANT TITLE: Role and Regulation of Emulsifier Production  
in Microbial Degradation of Heavy Hydrocarbons

REPORTING PERIOD: 1 April 1991 - 1 July 1991

AWARD PERIOD: 1 April 1991 - 31 March 1994

OBJECTIVE: Study the role and regulation of emulsifier production in attachment to and degradation of heavy hydrocarbons (lubricating oils and fuels) by strains of *Acinetobacter calcoaceticus*.

ACCOMPLISHMENTS: This is a new grant.

ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 4412207

PRINCIPAL INVESTIGATOR: Steven F. Karel

INSTITUTION: Princeton University

GRANT TITLE: Control of Supplementary Source Feeding in  
Biodegradation of Halogenated Aromatic  
Compounds

REPORTING PERIOD: 1 April 1991 - 1 July 1991

AWARD PERIOD: 1 April 1991 - 31 March 1994

OBJECTIVE: Determine the effect to alternate carbon sources  
on the regulation of biodegradation pathways for halogenated  
aromatic compounds.

ACCOMPLISHMENTS: This is a new grant.

ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 4412208

PRINCIPAL INVESTIGATOR: Mary E. Lidstrom

INSTITUTION: California Institute of Technology

GRANT TITLE: Bioremediation of Trichloroethylene by Marine  
Methanotrophs

REPORTING PERIOD: 1 April 1991 - 1 July 1991

AWARD PERIOD: 1 April 1991 - 31 March 1994

OBJECTIVE: Develop molecular methods for determining in situ populations of methanotrophs and assess the role of methanotrophs in biodegradation of TCE in samples from near-shore environments. Investigate effects of alternate nutrients on TCE metabolism of methanotrophs.

ACCOMPLISHMENTS: This is a new grant.

ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 4412209

PRINCIPAL INVESTIGATOR: Mary Ann Moran

INSTITUTION: University of Georgia Research Foundation,  
Inc.

GRANT TITLE: Potential for In Situ Aromatic Pollutant  
Remediation by Lignin-Degrading Marine  
Actinomyces

REPORTING PERIOD: 1 April 1991 - 1 July 1991

AWARD PERIOD: 1 April 1991 - 31 March 1994

OBJECTIVE: Determine the ability of lignin degrading  
actinomyces from marine environments to degrade aromatic  
pollutants.

ACCOMPLISHMENTS: This is a new grant.

ANNUAL PROGRESS REPORT

GRANT #: R&T CODE: 4412210

PRINCIPAL INVESTIGATOR: Stephen W. Ragsdale

INSTITUTION: University of Wisconsin

GRANT TITLE: Enzymology of Pathways for Anaerobic  
Degradation of Aromatic and Chlorinated  
Compounds

REPORTING PERIOD: 1 April 1991 - 1 July 1991

AWARD PERIOD: 1 April 1991 - 31 March 1994

OBJECTIVE: Enzymes responsible for anaerobic of aromatic compounds such as methoxylated aromatics, toluene, benzene, and phenylacrylate will be purified and mechanisms will be studied. Mechanisms of reductive dehalogenation by corronoids and corronoid proteins will be elucidated. (Corronoids contain Co).

ACCOMPLISHMENTS: This is a new grant.



ANNUAL PROGRESS REPORT

GRANT #: R&T CODE: 4412211

PRINCIPAL INVESTIGATOR: Juergen Wiegel

INSTITUTION: University of Georgia Research Foundation,  
Inc.

GRANT TITLE: Characterization of Selected Bacteria and  
Enzymes Involved in the Sequential, Anaerobic  
Degradation of 2,4-Dichlorophenol

REPORTING PERIOD: 1 April 1991 - 1 July 1991

AWARD PERIOD: 1 April 1991 - 31 March 1994

OBJECTIVE: Isolate an anaerobic 2,4-dichlorophenol-  
dehalogenating organism and purify the 2,4-dichlorophenol  
dehalogenase. Characterize the degradation pathway from  
phenol to benzoate.

ACCOMPLISHMENTS: This is a new grant.

## **EDUCATION AND TRAINING**

### **CORE PROGRAM**

**SCIENTIFIC OFFICERS: DRS. RANDALL S. ALBERTE AND ERIC EISENSTADT**

**PROGRAM OBJECTIVES: TO PROVIDE PROMISING YOUNG SCIENTISTS AT THE GRADUATE AND POST-DOCTORAL LEVELS WITH STATE-OF-THE-ART TRAINING IN RESEARCH AREAS SUMMARIZED IN THIS DOCUMENT AND TO INCREASE THE NUMBER OF MINORITY STUDENTS WHO CHOOSE BIOLOGY AS A PROFESSION.**

**NAVY OBJECTIVE: TO MEET NAVAL AND NATIONAL MANPOWER NEEDS IN SCIENCE AND TECHNOLOGY.**

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1978

R&T CODE: 4412083

PRINCIPAL INVESTIGATORS: Celia Bonaventura, Ph.D.  
Rebecca J. Van Beneden, Ph.D.

INSTITUTION: Duke University Marine Laboratory  
Beaufort, NC 28516

GRANT TITLE: Advanced Research Training in Marine  
Molecular Biology and Biotechnology

REPORTING PERIOD: May 1, 1990 - April 30, 1991 (12 months)

AWARD PERIOD: May 1, 1990 - April 30, 1992

OBJECTIVE: To train undergraduate/graduate level candidates in an intensive program designed to teach basic principles and techniques of cellular and molecular biology and the application to marine organisms.

ACCOMPLISHMENTS (last 12 months: The course last summer (June 18-July 20, 1990) was highly successful and rewarding and well organized for a "first offering". The laboratory participants were limited to 10. Student evaluations indicated that they were very pleased with the general format. The experiments allowed for a lot of hands-on work by the students and for a feeling of accomplishment as well. They especially liked the combination of field work and laboratory experimentation. We were able to cover a great deal of material during the 5 week semester. By using marine models and the tools of molecular biology, trainees obtained direct knowledge of indigenous marine organisms and discovered the unique properties that allowed them to be used as models in environmental and human health problems. Research methodology included DNA, RNA and protein isolation and manipulation; chromatographic methods; Southern, Northern and Western blotting; gene transfer techniques and CAT assays; gene amplification (PCR); DNA sequencing; gene cloning, library screening procedures and plaque purification; basic bacteriological and cell culture techniques; and gene mapping.

SIGNIFICANCE: We have kept in contact with many of the students. One undergraduate became very interested in oncogenes and is applying for graduate school this fall in this area. One student has taken a position as research assistant in a molecular biology laboratory at Johns Hopkins University and has future plans to enter graduate school studying marine organisms at this level. One assistant professor has incorporated molecular techniques in

her studies of marine snails. Two postdoctoral fellows are making excellent progress using recombinant DNA techniques and aquatic models and are planning publications in this area within the year. A predoctoral student will begin his studies soon and will incorporate some of the techniques he learned. These examples speak well of the far-reaching effects of such a course.

WORK PLAN (next 12 months): The lecture/lab course will be taught June 17-July 19, 1991, to 10 trainees. Independent study will be offered July 22-August 23, 1991, to trainees who have completed the course, making use of the techniques mastered during the course. Research methodology includes: Week 1 (Dr. Rebecca Van Beneden) - DNA isolation, Southern blotting, gene mapping and DNA sequencing; Week 2 (Dr. James Oliver) - basic bacteriology, characterization of marine bacteria, isolation of novel pharmaceuticals; Week 3 (Dr. Donald Blair) - cell culture, gene transfer techniques and CAT assays; Week 4 (Dr. Patricia McClellan-Green) - RNA isolation, cDNA cloning; Week 5 (Drs. Robert Chapman and Gary Wessel) - mt DNA isolation, PCR DNA amplification, in situ hybridization. The schedule has been modified slightly to help those students who have had little exposure to the experimental concepts of marine molecular biology and are unfamiliar with the vocabulary of the field and will allow more emphasis on mtDNA and PCR.

PUBLICATIONS AND REPORTS (last 12 months):

Course flyer announcing the availability of ADVANCED RESEARCH TRAINING IN MARINE MOLECULAR BIOLOGY AND BIOTECHNOLOGY.

ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 4412101

PRINCIPAL INVESTIGATOR: Michael I. Goldberg

INSTITUTION: American Society for Microbiology

GRANT TITLE: American Society for Microbiology Lecture (May 5-10, 1991)

REPORTING PERIOD: 15 March 1991 - 1 July 1991

AWARD PERIOD: 15 March 1991 - 1 July 1991

OBJECTIVE: Funds are provided to pay for the expenses (travel, lodging, honorarium, meals) of a distinguished microbiologist who will deliver the opening lecture to the Annual Meeting of the American Society for Microbiology. The lecture this year will be given by Dr. Robert L. Metzenberg of the University of Wisconsin Medical School.

ACCOMPLISHMENTS: This is a new grant.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-3245

R&T CODE: hbcu006-8903

PRINCIPAL INVESTIGATOR: Lynette P. Padmore

INSTITUTION: Florida A & M University

GRANT TITLE: BIONR/FAMU Center for Advancing Minority  
Participation in the Biological Sciences and Related  
Areas

REPORTING PERIOD: 30 September 1989 - 30 April 1994

AWARD PERIOD: 1 May 1991 - 30 April 1994

OBJECTIVE: In view of its critical dependence on advanced technology, the Navy has a very serious interest in helping to increase the number of U.S. citizens trained in science and engineering. This program is aimed at increasing the numbers and the quality of undergraduate, underrepresented minority students in science and engineering and, over the long term, to encourage their ability and enthusiasm to pursue graduate school in science and engineering.

ACCOMPLISHMENTS: Seven undergraduate scholars were identified and supported. Three were sophomores and four were freshman. One student spent last summer at Baylor College of Medicine. Five of the scholars participated in an intense two week academic enhancement program between the spring and summer semesters. Thirty six high school students participated in two precollege programs designed to stimulate learning. This program was called the Molecular Biology Institute. Student evaluations for all programs have rated activities very high.

ANNUAL PROGRESS REPORT

GRANT #:

R&T CODE: 4412099

PRINCIPAL INVESTIGATOR: Donal T. Manahan

INSTITUTION: University of Southern California

GRANT TITLE: Biochemistry and Molecular Biology of Marine  
Organisms

REPORTING PERIOD: 1 March 1991 - 1 July 1991

AWARD PERIOD: 1 March 1991 - 29 February 1994

OBJECTIVE: To train graduate and post-graduate students in  
new technologies and approaches to understanding marine  
organisms and biological oceanographic processes.

ACCOMPLISHMENTS: This is a new grant.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-4048

R&T PROJECT CODE: 4412088

PRINCIPAL INVESTIGATOR: James B. Stukes, Ph.D.

INSTITUTION: South Carolina State College

GRANT TITLE: Marine Science Initiative at South Carolina State College:  
An Investigation of the Biosensing Parameters Regulating  
Bacterial and Larval Attachment on Submerged Substrata

REPORTING PERIOD: 15 May 1990 - 14 May 1991

AWARD PERIOD: 15 May 1990 - 14 May 1993

OBJECTIVES: a) to determine the parameters required for the preferential settlement of marine bacterium, Pseudomonas fluorescens and the other bacteria (to be identified, TBI) associated with the cyprid larvae, Balanus eburneus and b) to determine the mechanism of chemosensory recognition of B. eburneus in the presence of extracellular polysaccharides produced by P. fluorescens and other bacteria, (TBI).

ACCOMPLISHMENTS (last 12 months): Research - We have successfully cultured and isolated extracellular polysaccharide (EPS) from P. fluorescens. Results indicate that both free EPS and cell-associated EPS consist of molecular weight sugars of 300,000 - 3,000,000 daltons as determined by Sepharose 4B size exclusion columns. However, the cell associated EPS has a higher concentration of total carbohydrate when compared to the total concentration for free EPS. Further, experiments involving the chemotaxis and attachment of P. fluorescens to polystyrene surfaces have been initiated. In addition, a culture system has been established in the laboratory in which adult barnacles (Balanus eburneus) spawn, and their larvae are reared to the cyprid stage. However, unlike B. amphitrite, B. eburneus cyprids do not readily settle on clean polystyrene surfaces. Cyprids up to two weeks in age settle at rates less than 5%. A high percentage of cyprids known as "floaters", move to air-water interface and are trapped on these surface films. Moreover, these cyprids are less likely to settle than nonfloaters.

Student training - During the summer of 1990, a summer marine science experience was established at the S.C. Wildlife and Marine Resources Department (SCWMRD) and S.C. State College (SCSC). While at SCWMRD, three students had an opportunity to be exposed to varying experiences in Marine Science, i.e., seabird census, pathology and histology of samples of clams, oysters, and fish, clam and finfish mariculture, benthic ecology, estuarine communities, and sediment contaminants. While at S.C. State College, the students learned a number of research techniques and methods which included growth and maintenance of bacteria, statistics, purification of the bacterial extrudants, and techniques of chromatography, microscopy and spectrophotometry. The summer experience stimulated the students to develop their research and critical thinking skills and to gain an appreciation of the various environmental factors which influence marine ecological systems. In addition, during the spring semester of 1991, a course entitled, "Concepts in Marine Science" was reintroduced as a team taught course at SCSC, involving all the scientists in the project. The course was very successful in exposing students to various concepts in Marine Science, and most importantly, in recruiting students to participate in the ONR research/training program. Three of the five students who were enrolled in the course will be participating in the ONR program beginning on June 10, 1991.

SIGNIFICANCE: The analyses of the extracellular polysaccharides of



marine bacteria will provide us with information concerning their composition and their role in the attachment of bacteria and barnacles to substrata. The control of EPS production has application in marine environments where fouling organisms are a problem and the attachment or detachment of these organisms has economic importance to the Navy. Further, we are interested in increasing the alarmingly small pool of minority students interested in pursuing careers in marine science through a coordinated research training program.

WORK PLAN (next 12 months): Research - The objectives for the next 12 months are to further characterize the extrudants to determine the percentage of protein, pyruvate, and acetyl, in addition to total carbohydrates. The extrudants will also be tested to determine their role in attachment of bacteria and barnacles to substrata. Further, to determine whether larval B. amphitrite reared under the conditions at the SCWMRD laboratory will settle on polystyrene surfaces, as they do in Dr. Rittschof's laboratory. This test will assess whether culture conditions are adequate for producing competent larvae, or if the requirements for the settlement of B. eburneus are indeed different for B. amphitrite.

The relative abundance of P. fluorescens and other bacteria associated with B. eburneus on natural substrata will also be determined. If other species of bacteria are of greater ecological relevance, these bacteria will be used in the laboratory bioassay experiments at SCWMRD and in extrudant analyses at SCSC. Moreover, to meet the goals of both cooperative institutions, the budget will be restructured, and additional funds from ONR may be requested.

Student training - The student training component during the summer of 1991 will begin on June 10th and end July 19th, and will involve 8 undergraduate students rather than 3, during the six weeks summer experience (SCWMRD - 3 wks and SCSC - 3 wks). We are really excited about this summer because we are hoping to improve the integration of the summer experiences at SCWMRD and SCSC. To achieve this goal, faculty members from SCSC will actively participate in the summer experience at the SCWMRD with the SCSC students. We feel this involvement of the SCSC faculty members will enhance the summer experience by ensuring that the students have a mentor with whom they are familiar and with whom they can relate in a new environment. Further, samples that the SCSC students and faculty members collect at various sites, i.e., Charleston Harbor, Waddell Mariculture Center, and the ACE Basin will be examined in the laboratory at S.C. State College. Scientists from SCWMRD will assist the students and SCSC faculty members with the analysis of collected samples. In addition, a SCSC student will assist Dr. Nancy O'Connor with her barnacle studies at SCWMRD. We feel that the overlapping participation of SCSC and SCWMRD scientists will make this summer experience very enriching.

PUBLICATIONS AND REPORTS (last 12 months): None. However, we are very optimistic that within the upcoming months, we will generate sufficient data for preparation and submission of a manuscript.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1463

R&T CODE: 4412077

PRINCIPAL INVESTIGATOR: A.O. Dennis Willows

INSTITUTION: University of Washington

GRANT TITLE: Advanced Training in Molecular Marine Biology

REPORTING PERIOD: 1 Dec 1989 - 30 Nov 1991

AWARD PERIOD: 1 Dec 1989 - 30 Nov 1991

OBJECTIVE: To initiate, organize and offer a marine molecular neurobiology course at a graduate level to about 12 students drawn from a national/international pool.

ACCOMPLISHMENTS (last 12 months): The course Zoology 538b (Marine Molecular Neurobiology) was organized and offered in May-June of 1990 and again in 1991. The faculty and students are named in the attached information. The ONR funding was crucial to this insofar as it provided equipment, some materials and supplies, fellowship support for some of the students, and part of the cost of importing an eminent faculty support group.

SIGNIFICANCE: The course is a new offering for the FHL, and perhaps for the country. It focusses upon presentation of the basic techniques of molecular biology, and neurobiology and also introduces participants to the rich faunal opportunities of the marine environment. It is an unusual, perhaps unique combination, which offers interdisciplinary approaches to some of the toughest problems in neuroscience.

WORK PLAN (next 12 months): The course is being offered again in 1991. It is anticipated that it will become a long-term, if not permanent component of the FHL curriculum.

PUBLICATIONS AND REPORTS (last 12 months): The students work in pairs upon weekly projects and write up (and present) their results to the rest of the group. Their written reports are gathered together, and bound into a book which is accessed to the University of Washington Library. That report is a permanent part of the University Library and is available on interlibrary loan.

**HIGH TEMPERATURE AND PRESSURE BIOLOGY**

**CORE PROGRAM (OUTGROWTH OF AN ARI JOINT WITH OCEANIC BIOLOGY  
ON ARCHAEABACTERIA)**

**SCIENTIFIC OFFICER: DR. ERIC EISENSTADT**

**PROGRAM OBJECTIVE: TO DETERMINE MOLECULAR MECHANISMS RESPONSIBLE  
FOR THE ABILITY OF ORGANISMS TO ADAPT TO AND MAKE A LIVING AT HIGH  
TEMPERATURES & PRESSURES.**

**NAVY OBJECTIVE: TO DEVELOP TECHNOLOGIES FOR MAKING DEVICES THAT  
WILL PERFORM AS BIOSENSORS, SURFACTANTS, STRUCTURAL POLYMERS, AND  
CATALYSTS IN HARSH NAVAL ENVIRONMENTS.**

## ANNUAL PROGRESS REPORT

GRANT#: N00014-90-J-1894

R&T CODE: 4412084-03

PRINCIPAL INVESTIGATOR: Michael W. W. Adams

INSTITUTION: University of Georgia

GRANT TITLE: "The Metabolism of C-1 Compounds by Extremely Thermophilic Bacteria"

REPORTING PERIOD: 1 June 1990 - 31 May 1991 (12 months)

AWARD PERIOD: 1 June 1990 - 31 May 1993

OBJECTIVE: Extremely thermophilic marine bacteria are only distantly related to all other organisms, considered by some as a separate kingdom of life, and their extraordinary growth characteristics have raised fundamental biological questions. For example, how do they stabilize various biomolecules, such as proteins, cofactors and numerous metabolites, at temperatures near and above 100°C? Do they have use the conventional metabolic pathways found in mesophilic bacteria, or do completely different pathways exist that have taken advantage of the unique ecosystems in which they are found? The overall aims of our ONR-funded research are to try provide some answers to such questions, by studying the metabolism of carbon compounds such as CO, formate, and CO<sub>2</sub>, in these organisms.

ACCOMPLISHMENTS (last 12 months): The main focus has been the elucidation of a novel pathway of CO<sub>2</sub> production during carbohydrate catabolism, primarily in *Pyrococcus furiosus*, and also in other extremely thermophilic archaeobacteria such as *Thermococcus litoralis*, *Pyrodictium abyssum*, and the as yet unclassified "ES-4", all of which grow optimally near and above 100°C. We have shown that the growth of these organisms is dependent upon tungsten (W), an element seldom used in biological systems, and a novel W-containing aldehyde ferredoxin oxidoreductase has been purified from *P. furiosus*. This is proposed to be a key enzyme in an unusual "pyroglycolytic" pathway in which glucose is converted to acetate, H<sub>2</sub> and CO<sub>2</sub>, without the use of NAD(P). All of these organisms require both peptides and carbohydrates for optimal growth, but the relationship between their metabolisms is unclear. We have now identified two key enzymes in *P. furiosus* that link peptide catabolism and the novel glycolytic pathway. They are glutamate dehydrogenase, which converts glutamate to 2-ketoglutarate, and 2-ketoglutarate oxidoreductase, which oxidizes the keto acid to produce CO<sub>2</sub>, succinyl CoA, which could feed into the glycolytic pathway, reduced ferredoxin, which couples to the hydrogenase to produce H<sub>2</sub>. We are also investigating the mechanisms of extreme thermostability of the proteins from these organisms. Specifically, we are attempting to obtain a high resolution

three-dimensional structure of *Pyrococcus furiosus* rubredoxin, a small and 'hyperthermostable' redox protein that we have purified and crystallized, using 2D NMR and X-ray protein crystallography. Preliminary NMR data show that the protein has extensive secondary structure, and that its N-terminal residue forms a specific ionic bridge that is not found in thermolabile mesophilic rubredoxins.

SIGNIFICANCE: Our results are providing the first insights into how extremely thermophilic bacteria obtain energy from the co-metabolism of C and N compounds, and how this can be achieved at high temperature. For example, they appear to have a novel glycolytic pathway based on the rarely used element tungsten, in which carbohydrates are oxidized to  $H_2$  and  $CO_2$  without using the thermolabile cofactors, NAD or NADP. Our studies are also providing the first information on the mechanisms by which these organisms stabilize proteins. In the case of the hyperthermostable rubredoxin, this will be the first protein whose three-dimensional structure is independently elucidated by three different techniques: by crystallography, by molecular modelling, and by NMR.

WORK PLAN (next twelve months): The objectives in the next year are to a) further characterize the novel "pyroglycolytic pathway" pathway, b) to purify and characterize the enzyme 2-ketoglutarate oxidoreductase from *P. furiosus*, c) to examine other extreme thermophiles such as *Hyperthermus butylicus*, to see if their growth is also dependent upon tungsten and to analyze for the proposed pathway, and d) to continue structural studies of the hyperthermostable rubredoxin from *P. furiosus*.

PUBLICATIONS AND REPORTS (last twelve months):

1. Makund, S. and Adams, M. W. W. (1991) "The novel tungsten-iron-sulfur protein of the hyperthermophilic archaeobacterium, *Pyrococcus furiosus*, is an aldehyde ferredoxin oxidoreductase: evidence for its participation in a unique glycolytic pathway" *J. Biol. Chem.* (August issue)

2. The following paper was submitted to *Biochemistry*:

Blake, P. R., Park, J. B., Bryant, F. O., Aono, S., Magnuson, J. K., Eculston, E., Howard, J. B., Summers, M. F. and Adams, M. W. W. (1991) "Determinants of protein hyperthermostability. 1. Purification, amino acid sequence, and secondary structure from NMR of the rubredoxin from the hyperthermophilic archaeobacterium, *Pyrococcus furiosus*"

Aspects of this research were presented at invited seminars at Argonne National Laboratories, IL, Stratagene, La Jolla, CA, Cornell University, NY, and at the Annual Meetings of the American Chemical Society and the American Society of Microbiology, both held in Atlanta, GA.

## ANNUAL PROGRESS REPORT

CONTRACT#: N00014-90-J-1878

R&T CODE: 4412086

PRINCIPLE INVESTIGATOR: Douglas Bartlett

INSTITUTE: Scripps Institution of Oceanography

CONTRACT TITLE: Pressure Regulation in Deep Sea Bacteria

PERIOD OF PERFORMANCE: 1 June 1990 - 1 June 1991

**OBJECTIVE:** Pressure is an essential thermodynamic parameter which has profound effects on biological processes in the deep ocean. Unlike terrestrial organisms, deep sea organisms are adapted to elevated pressures, and may sense and acclimate to alterations in their pressure regimes. We are using the barophilic bacterium *Vibrio* SS9, and the pressure unadapted bacterium *Escherichia coli* as model systems in which to study the effects of elevated pressure on high pressure adapted and high pressure sensitive bacteria. Our principle first year objectives have been to: A) isolate regulatory mutants in *Vibrio* SS9 impaired in their ability to regulate the pressure inducible protein OmpH. B) Assess the physiological role of the OmpH protein. C) Examine the stress proteins induced in *E. coli* as a result of high pressure treatments. D) Attempt the isolation of pressure sensitive mutants in *Vibrio* SS9.

**PROGRESS REPORT:** A) We have employed the *lacZ* gene from *E. coli* as a reporter gene for monitoring pressure inducible *ompH* transcription. A promoter-less *lacZ* containing DNA fragment was cloned into the 5' end of the *ompH* gene such that the *ompH* promoter would direct the transcription of the *lacZ* gene resulting in the production of the readily assayed *lacZ* gene product,  $\beta$ -galactosidase. After transferring the *ompH::lacZ* fusion onto a broad host range plasmid, the DNA was conjugated into a Lac<sup>-</sup> mutant of SS9 and a double recombinant resulting in the integration of the fusion into the deep sea bacterial chromosome was isolated. This strain, designated EC10, was found to display pressure inducible  $\beta$ -galactosidase production. These results indicated that pressure is regulating the abundance of the *ompH* mRNA at the level of transcription initiation. EC10 was mutagenized with the frameshift mutagen ICR191 and mutants displaying diminished  $\beta$ -galactosidase production were isolated. Two of these mutants were found to contain mutations that mapped outside of the *ompH* locus by complementation analyses. Both of these mutants produced little  $\beta$ -galactosidase at either low or high pressure, and were defective in the production of two additional proteins which are typically pressure inducible in SS9. These results suggest that we have isolated mutations at a regulatory locus (or loci) which controls multiple pressure inducible genes.

B) What is the function of OmpH? We have found that OmpH<sup>-</sup> mutants grow poorly in minimal marine media; adding more credence to the notion that OmpH is a porin protein. However, in vitro analyses of OmpH diffusion channel activity in artificial membranes (performed by a collaborator: Dr. Lucas Buehler) have not been conclusive owing to the presence of additional porin species in our OmpH preparations.

C) We are also studying the effects of pressure on "stress" protein production in the pressure sensitive bacterium *E.coli*. Pulse-labelling experiments have revealed that elevated pressure induces the production of a number of specific proteins in *E.coli*. For example, after 2 hours at 560 atm. approximately 6 unique proteins are strongly induced, while after 2 hours at 1120 atm. only two very low abundance protein species are still observed. 2-D gel analyses are aiding protein species identifications.

D) We are interested in isolating pressure sensitive SS9 mutants because of their great potential in identifying key cellular processes which are important for pressure adaptation. However, it is not currently possible to screen large numbers of bacterial clones at high hydrostatic pressure. Therefore, we first sought to isolate certain conditional mutants which might also display altered pressure growth profiles. Unfortunately, after screening 70 cold sensitive, and 22 temperature sensitive SS9 mutants, none were found to be altered in their pressure adaptation.

**WORK PLAN** (year 2): A) We will complement the *ompH* regulatory mutants using an SS9 broad host range cosmid library. DNA responsible for complementation will be mapped and sequenced. Later a broad host range T7 promoter system will be used to overproduce and localize the regulatory proteins within SS9. These experiments will allow us to develop models to describe pressure sensing and pressure regulation of gene expression in SS9. B) OmpH will be purified away from contaminating porin proteins and its activity in vitro will be monitored. C) The pressure stress proteins induced in *E.coli* will be identified by performing additional stress response experiments and using *E.coli* mutants defective in the production of specific stress proteins. We will expand our pulse labelling of proteins synthesized under pressure to include SS9 and perhaps other deep sea bacteria. D) In collaboration with Dr. Douglas Clark at the Univ. Cal. at Berkeley we will employ elevated hyperbaric pressure to screen for SS9 pressure sensitive mutants. We will also select for increased pressure tolerance in both SS9 and *E.coli*.

**TRAINING ACTIVITIES:** The doctoral studies of Ellen Chi are supported by our ONR contract, as are supplies for Leandro Arca, and Susan Slaughter, both undergraduate students.

**PUBLICATIONS** (last 12 months):

Chi, E., and D. H. Bartlett. 1991. Pressure regulation of *ompH* transcription in the Deep Sea Bacterium SS9. Abstract. American Society for Microbiology National Meeting. p. 198.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-1900

R&T CODE: 4412062

PRINCIPAL INVESTIGATOR: Dr. Robert Blake II

INSTITUTION: Meharry Medical College

CONTRACT TITLE: Respiratory Enzymes of Sulphobus brierlevi

REPORTING PERIOD: 1 June 1990 - 30 May 1991

AWARD PERIOD: 1 July 1989 - 30 September 1991

OBJECTIVE: (1) To develop an improved procedure for the routine large scale culture of archebacterial iron autotrophs based on the in situ electrolysis of the soluble iron in the growth medium; and (2) to initiate the identification, separation, purification, and characterization of the individual cellular components that comprise the iron respiratory electron transport chain.

ACCOMPLISHMENTS: (last 12 months): Aerobic respiration on Fe(II)  
An in situ electrolytic procedure was developed whereby large quantities of autotrophic iron oxidizing archebacteria were achieved on a reproducible basis. Typical yields of Acidianus (formerly Sulfolobus) brierlevi increased from 20 to 400 mg wet weight/liter, while those of Metallosphaera sedula increased from 50 to 500 mg wet weight/liter. A novel acid-stable cytochrome was solubilized from both A. brierlevi and M. sedula by sonic oscillation of whole cells in the presence of the ionic detergent Zwittergent-12. The solubilized cytochrome was then purified until it constituted one major band by SDS-PAGE. The highly purified cytochrome was rapidly reduced by soluble Fe(II) at pH 2.0. The absorbance properties of this cytochrome (particularly the reduced peak at 572 nm) distinguished it as a unique electron character not previously described in other bacteria that respire on soluble ferrous ions.

### Aerobic respiration on reduced arsenic

Research activities were limited to a continuation of the descriptive characterization of the unnamed microorganism (ATCC 53921) that grew autotrophically on metal arsenides. The bacterial-dependent oxidation of gallium arsenide proceeded equally well whether the inorganic substrate was presented as a highly purified reagent, as crude saw "kerf", as macerated water scrap material (either doped or undoped), or as an integral surface component of intact, defective integrated circuit chips. Environmental scanning electron microscopic examinations of gallium arsenide wafers inoculated with ATCC 53921 revealed numerous cocci associated with the biologically-eroded surface. The diameter of the cocci varied from 0.5 to 1.0 microns. Efforts to achieve the large scale culture of ATCC 53921 in the absence of arsenide-containing materials were unsuccessful.

SIGNIFICANCE: The development of an improved procedure to obtain high yields of thermophilic iron autotrophs is a significant technological advance that will facilitate future investigations of these difficult-to-culture organisms. The preliminary characterization of the novel cytochrome from M. sedula provides the first evidence that archebacteria that respire on iron possess respiratory chain components that have not been described in the corresponding eubacteria.

### WORK PLAN (next 12 months): Aerobic respiration on Fe(II)

The principal objectives next year are (i) to complete the purification of the novel cytochrome from M. sedula to electrophoretic homogeneity and (ii) to subsequently initiate a detailed investigation of the structural and functional properties of the purified protein. Protein



sequencing and stopped flow spectrophotometric activities will be performed. Efforts to identify and isolate other respiratory chain components from A. brierleyi and M. sedula will continue.

**Aerobic respiration on reduced arsenic**

The principal objective next year is to devise cell culture protocols whereby sufficient masses of ATCC 53921 may be achieved to permit the initiation of taxonomic classification and biochemical characterization activities. Efforts will focus on the identification of an inexpensive growth substrate that does not interfere with standard cell harvesting techniques.

PUBLICATIONS AND REPORTS (last 12 months):

1. Two non-refereed papers on the bacterial transformation of metal arsenides were submitted.

Blake, II, R.C. and Bowers-Irons, G. (1991) "Bioremediation of metal arsenide wastes" in National Research and Development Conference on the Control of Hazardous Materials, Hazardous Materials Control Research Institute, Greenbelt, MD, pp. 330-333.

Blake, II, R.C. and Bowers-Irons, G. (1991) "Microbially influenced corrosion of metal arsenides" in Microbially Influenced Corrosion and Biodeterioration (Dowling, N., Mittelman, M. and Danko, J., eds.), University of Tennessee, Knoxville, TN, in press.

2. Two abstracts on the novel cytochrome from M. sedula were recently submitted.

Blake, II, R.C., Shute, E.A. and Greenwood, M. (1991) "Enzymology of Respiratory Iron Oxidation", submitted to SIM annual meeting in Philadelphia in August.

Blake, II, R.C., Shute, E.A., Greenwood, M. and Spencer, G.H. (1991) "Enzymology of Respiratory Iron Oxidation", submitted to Biohydrometallurgy '91 to be held in Portugal in September.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1884

R&T CODE: 441d024

PRINCIPAL INVESTIGATOR: Douglas S. Clark

INSTITUTION: University of California at Berkeley

GRANT TITLE: Pressure-Temperature Effects on Thermophilic  
Archaeobacteria

REPORTING PERIOD: 1 July 1990 - 30 May 1991

AWARD PERIOD: 1 March 1989 - 30 September 1991

OBJECTIVES: To examine pressure-temperature relationships in the behavior of extreme thermophiles isolated from submarine hot vents; to compare the effects of pressure on deep-sea and shallow-water organisms; to determine the properties of hydrogenases and proteases isolated from thermophilic methanogens as a function of temperature and pressure.

ACCOMPLISHMENTS (last 12 months): Growth and metabolism of the marine archaeobacterium ES4 [Pledger and Baross, J. Gen. Microbiol., 137, 203 (1991)] were studied at elevated (hyperbaric) pressures. Substantial differences were observed when ES4 was incubated in a stainless steel versus glass-lined pressure vessel. In the stainless steel vessel, metabolic CO<sub>2</sub> production was measured at temperatures of 85 °C, 95 °C, 100 °C, 105°C at total pressures of 8, 250, and 500 atm. H<sub>2</sub> and H<sub>2</sub>S were also produced but the large amounts of helium at 250 and 500 atm precluded measurement of these gases at higher pressures. From 85 °C to 100 °C, CO<sub>2</sub> production increased slightly as the pressure was increased from 8 to 250 atm, and increased substantially when the pressure was increased to 500 atm. At 105 °C, no gas was produced until the pressure was increased to 500 atm. Epiflorescent microscopy showed that cell numbers did not change over the time period that gas production was measured.

A second series of experiments in a glass-lined vessel compared the specific growth rate of ES4, as measured by cell production, at 8 and 250 atm. A new liquid sampling system was used which allowed decompression of liquid samples before removal from the pressure vessel. Decompressing the samples reduced the rate of cell lysis upon sampling from 250 atm by ten-fold. At temperatures below 95°C, a pressure of 250 atm caused a minor reduction in growth rate, whereas from 95 to 98 °C, pressure had no noticeable effect on either growth or CO<sub>2</sub> production.

The thermal stabilities of the hydrogenases from *M. jannaschii* are being studied as a function of pressure. The F<sub>240</sub>-nonreactive hydrogenase was separated from the F<sub>240</sub>-reactive hydrogenase and examined at 10 atm and 500 atm. Deactivation of the enzyme at 90 °C was retarded by pressure: the half-lives at 10 and 500 atm were 0.55 hr and 2.39 hr, respectively.

Proteolytic activity has been detected in cell extracts of *M. jannaschii*. Initial studies revealed activity at temperatures ranging from 70 °C to 130°C, with a maximum at approximately 120 °C. The half-life at 110 °C was 2.5 hr.

SIGNIFICANCE: In stainless steel, CO<sub>2</sub> production by ES4 was accelerated by hyperbaric pressure under conditions of no growth. A similar phenomenon was observed at supraoptimal temperatures for the deep-sea thermophile *M. jannaschii*. Thus, barophilic metabolism in the absence of growth (e.g., at temperatures well above the optimal growth temperature) may be a distinguishing characteristic of many deep-sea vent archaeobacteria.

In general, the behavior of ES4, i.e., its ability to grow and its

response to elevated pressure, depends dramatically on its environment. Thus, depending on the experimental conditions, the behavior of the deep-sea thermophiles in the laboratory may be very different from that at hydrothermal vent sites.

High pressure has a potent stabilizing effect on  $F_{420}$ -nonreactive hydrogenase from M. jannaschii. Thus, pressure may be a general stabilizer of proteins from deep-sea and/or barophilic organisms. On the other hand, hydrogenases from M. jannaschii may be unique in their response to pressure.

WORK PLAN (remainder of the grant period): The newly isolated strain ES4 will be grown at 500 atm to determine if either growth or metabolism is enhanced at this pressure.

Pressure-induced stabilization of hydrogenases from M. jannaschii will be further studied at higher temperatures. Pressure-temperature studies of  $F_{420}$ -reactive hydrogenase in soluble and membrane-bound forms are in progress, as are studies of hydrogenase(s) from M. igneus and M. thermolithotrophicus.

PUBLICATIONS AND REPORTS (last 12 months):

Clark, D.S. and Kelly, R.M. (1990) "Hot Bacteria", Chemtech (invited paper) 20, 655.

Ludlow, J.M. and Clark, D.S. (1991) "Engineering Considerations for the Application of Extremophiles in Biotechnology," in Critical Reviews in Biotechnology, 10, 321.

Nelson, C.M. and Clark, D.S. "Pressure-Temperature Effects on the Growth and Metabolism of ES4, a Deep-Sea Vent Archaeobacterium," in preparation.

Nelson, C.M., Hei, D., Michels, P., and Clark, D.S. "Pressure-Temperature Effects on the Structure and Function of Enzymes from Deep-Sea Thermophiles," to appear in the ACS Symposium Series (proceedings from "Biocatalysis Near or Above 100 °C," ACS National Meeting, Atlanta, GA, April, 1991), in preparation.

Nelson, C.M., Hei, D., Michels, P., and Clark, D.S. "Pressure-Temperature Effects on the Structure and Function of Enzymes from Deep-Sea Thermophiles," lecture given at the ACS National Meeting, Atlanta, GA, April, 1991.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-j-3077

R&T CODE: 4412012

PRINCIPAL INVESTIGATOR: Dr. Charles J. Daniels

GRANT TITLE: Substrate Recognition by the Archaeobacterial tRNA  
Intron Processing Enzymes.

PERIOD OF PERFORMANCE: 1 July 1990 - 1 June 1991

OBJECTIVES: Identify the in vitro substrate recognition properties of the archaeobacterial tRNA intron endonuclease; develop plasmid based expression vectors for the introduction of genes into Halobacterium volcanii, use expression vectors to examine the in vivo processing of transcripts from modified intron-containing tRNA genes; develop methods to isolate mutants deficient in tRNA processing.

### ACCOMPLISHMENTS (YEAR 2):

In vitro Substrate Recognition by the Intron Endonuclease: We have established that the H. volcanii tRNA intron endonuclease requires both cleavage sites to be present in three nucleotide bulge loops, and that these loops be separated by four base pairs. These structural features are sufficient for recognition since non-tRNA model substrates, containing only these features, are substrates for the enzyme. Identification of the cleavage sites appears to utilize a measurement mechanism which senses the distance between the two cut sites. Contacts with specific nucleotides may also play some role in recognition; sequence changes in the four base pairs separating the two cut sites decreases cleavage efficiency. We have extended our characterization of this enzyme to the methanogens and Thermoplasma. These enzymes exhibit substrate recognition properties similar to the halobacterial enzyme. We have also observed that the halobacterial, methanogen, and Thermoplasma endonucleases are capable of cleaving the Desulfurococcus mobilis rRNA intron-containing precursor. These studies support our hypothesis that intron processing enzymes of the archaeobacteria are different from those found in the eukaryotes, and that this activity may represent a novel RNA restriction-like enzyme.

Related to these studies, we have made significant progress in the purification of the Methanosarcina barkeri and Thermoplasma tRNA endonucleases. Both endonucleases appear to be approximately 55 kD monomeric proteins.

Development of Plasmid Based Expression Vectors for the Halophiles and in vivo Processing Studies: During this past year we have made further modifications to the E. coli-H. volcanii shuttle expression vector constructed in year one (addition of a transcription termination signal), and begun the construction of modified intron-containing tRNA genes for in vivo analysis of tRNA processing. We have prepared expression plasmids with intron-containing tRNA<sup>Trp</sup> genes carrying the splicing defects determined from in vitro experiments. Additional mutations were introduced into the exon regions of these tRNAs (tagged exons) so that transcripts from these genes could be distinguished from those originating from the chromosome. We are currently evaluating the processing of these RNAs. We have also used this vector to express a eukaryotic intron-containing pre-tRNA gene, Saccharomyces cerevisiae

tRNA<sup>Pro</sup>, in H. volcanii. According to our in vitro model, the primary transcript of this gene should not be processed by the halobacterial splicing enzymes since it lacks the required structural elements for endonuclease cleavage. Northern and primer extension analysis verified the expression of this tRNA and demonstrated that, as predicted, intron processing did not occur in vivo. We have also noted that plasmids containing the tRNA<sup>Lys</sup> promoter are present in lower copy number than plasmid controls. This suggests that highly active promoters are selected against during transformation. We are now characterizing low copy mutants of this plasmid.

Isolation of tRNA Processing Mutants: Continuing with our exploration of methods for the isolation of RNA processing mutants, we have recently found that bacitracin may be an effective agent for enrichment of temperature sensitive mutations. Using a 24 hour bacitracin treatment at the nonpermissive growth temperature (45°C), we were able to increase the frequency of ts cells in the population by a factor of ten. Unfortunately, of 15 mutants screened, none were defective in intron processing. The use of an antisense "killer" RNA, directed against the HisC mRNA, as a selective agent is still being evaluated. When this "killer" RNA (presented as a tRNA<sup>Trp</sup> derivative) is bound to the HisC mRNA it will form a structure identical to the intron endonuclease preferred structure. We anticipate that the antisense RNA will render the cells His<sup>-</sup>, or slow growing in the absence of histidine. The feasibility of this approach will be known within the next few months.

WORK PLAN (Year 3): In the upcoming year of we will continue in our attempts to purify the endonuclease from the methanogens and Thermoplasma. The purified protein will be helpful in further analysis of substrate recognition and possibly provide a tool for the isolation of the endonuclease gene. Since the methods are now in place to analyze intron processing in vivo, we will be able to determine whether a defined structure is required in vivo. The ability to obtain RNA processing mutants is still the most speculate portion of this proposal. However, the possibility of using an enrichment technique should aid our screening efforts.

#### PUBLICATIONS AND REPORTS:

1. Thompson, L.D. and Daniels, C.J. 1990. Recognition of Exon-Intron Boundaries by Halobacterium volcanii tRNA intron endonuclease. J. Biol. Chem., 265: 18104-18111.
2. Nieuwlandt, D.T and Daniels, C.J. 1990. An Expression Vector for the Archaeobacterium Haloferax volcanii. J. Bacteriol., 172: 7104-7110.
3. Nieuwlandt, D.T, Haas, E.S., and Daniels, C.J. 1991. The RNA Component of RNaseP from the Archaeobacterium Haloferax volcanii. J. Biol. Chem., 266: 5689-5695.

## ANNUAL PROGRESS REPORT

GRANT#: N00014-91-J-1294

R&T CODE: .441D14--05

PRINCIPAL INVESTIGATOR:

W. Ford Doolittle

INSTITUTION:

Dalhousie University

GRANT TITLE:

Genetic tools for archaeobacteria

REPORTING PERIOD:

1 February 1991 - 3 June 1991

AWARD PERIOD:

1 February 1991 - 31 January 1994

**OBJECTIVE:** To further characterize already established transformation and recombination processes in the halophilic archaeobacteria, and refine existing shuttle vectors to serve as expression vectors. To establish conditions for transformation of a thermophilic archaeobacterium (e.g. *Sulfolobus* sp.), isolate and map mutants, and prepare vectors from existing small replicons.

**ACCOMPLISHMENTS (first 4 months):** *Halobacterial expression vectors.* Beginning with our halobacterial/*E. coli* shuttle vector pWL102, we have derived an improved basic shuttle vector. We reduced the size of the mevinolin resistance (*Mev<sup>r</sup>*) insert from 3.5 kb to 1.8 kb, eliminated a number of restriction sites originally dispersed within pWL102, and inserted a new multiple cloning site cassette. This new 8.7 kb shuttle vector is called pWL104 (see fig. on highlight page).

As one approach to constructing an expresssion vector we are taking advantage of the up-promotor mutation which confers resistance to mevinolin by overexpressing the *Haloferax volcanii* HMGC*o*A reductase gene (*hmgA*). We have isolated a DNA fragment comprising 320 bp of sequence upstream from the *Mev<sup>r</sup> hm**gA* start codon and inserted this fragment just upstream from the multiple cloning site cassette in pWL104. As a second approach we have fused the *Hf. volcanii trpCBA* promoter and the first several coding bases of the *trpC* gene to the *E. coli lacZ* gene. We are now in the process of characterizing these constructions.

The *trpCBA* and *trpDFEG* clusters map to separate locations in the *Hf. volcanii* chromosome. We are beginning to analyze regulation of *trp* gene expression and coordination of expression between the two gene clusters by quantitating RNA transcription in wild type and mutant strains grown on various combinations of Trp pathway intermediates.

An *Hf. volcanii* host whose genome lacks the *trpCBA* cluster will be useful in studying regulation of *trp* gene expression and will prevent recombination between plasmid-borne *trp* genes and the genome. We are in the process of constructing such a strain using an approach which we previously applied to deleting the *trpB* coding sequence.

Recombination occurs between the *Mev<sup>r</sup>* insert of our shuttle vectors and the *hmgA* locus of the *Hf. volcanii* genome. A selectable marker lacking homology with the genome would be desirable. Eventhough *Haloarcula hispanica* can be easily transformed to *Mev<sup>r</sup>* by pWL102, we find that a non-replicating construct bearing the 3.5 kb *Mev<sup>r</sup>* insert does not transform *Ha. hispanica* to *Mev<sup>r</sup>*. This suggests that there is insufficient sequence homology for integration of the *Hf. volcanii*-derived marker into the *Ha. hispanica* genome. Based on this observation, we have selected spontaneous *Mev<sup>r</sup>* mutants of *Ha. hispanica* and *Haloarcula marismortui* and are now attempting to clone and

characterize these *Mev<sup>r</sup>* loci in the hope that one or both of them will confer *Mev<sup>r</sup>* on *Hf. volcanii* without being capable of integration into its genome. Both *Ha. hispanica* and *Ha. marismortui* are naturally resistant to trimethoprim (Tnp) whereas many halobacteria, including *Hf. volcanii*, are sensitive to this antibiotic. Therefore we are also attempting to clone the *Tnp<sup>r</sup>* loci for use as alternative non-homologous selectable markers in *Hf. volcanii*.

**Thermophile transformation.** Our work in this area has mainly involved simply setting up to work with thermophiles in our laboratory. We now have *Sulfolobus solfataricus* strains P1 (DSM1616), P2 (DSM1617), B12 (DSM5389) and *Sulfolobus acidocaldarius* (DSM639) growing.

We are now attempting to develop a strain with a selectable marker. Mevinolin resistance has proven very reliable as a selectable trait in our previous work with halophilic archaeobacteria, so we are investigating whether this system can be developed in *Sulfolobus* as well. We have shown that *S. solfataricus* P2 is sensitive to mevinolin at levels that are comparable to those which inhibit *Hf. volcanii*. However, we have so far not been successful in attempts to select a spontaneous *Mev<sup>r</sup>* mutant of strain P2.

If we are ultimately unable to select for a spontaneous *Mev<sup>r</sup>* *Sulfolobus* mutant, this may indicate that multiple promotor mutations are required to substantially elevate HMGC<sub>o</sub>A reductase levels. In that event site specific mutagenesis of the promotor of a cloned *Sulfolobus hmgA* locus might yield a highly expressed gene. We have found that a degenerate 20-mer oligonucleotide probe based on a highly conserved . . . region in *Hf. volcanii* and eukaryotic HMGC<sub>o</sub>A reductases hybridizes to a unique 4 kb Eco RI-Mlu I fragment of *S. solfataricus* P2 genomic DNA.

**SIGNIFICANCE:** Archaeobacterial expression vectors will be useful tools for research and biotechnology. Comparison of archaeobacterial *trp* gene regulation and expression with well characterized eubacterial models will result in a better understanding of the level of sophistication of gene regulation and expression in the last common ancestor of all living things. Characterization of the *hmgA* gene will contribute to our understanding of the early evolution of the eukaryotic kingdom.

**WORK PLAN (next 12 months):** In addition to continuing with the work in progress described above, we will attempt to express *E. coli lacZ*, *Halobacterium halobium* bacteriorhodopsin (*bop*) and *Vibrio harveyi lux* genes in *Hf. volcanii* as part of our characterization of the potential expression vectors.

We will sequence the potential *Sulfolobus hmgA* homologue and, if prospects seem favorable, site specifically alter its promotor. At the same time we will develop other selection systems for *Sulfolobus*, including auxotrophic mutants, in which successful transformation can be scored. Once we have a selectable marker to work with, we will begin developing a *Sulfolobus* transformation system, initially attempting transformation with genomic DNA. Both electroporation and PEG transformation systems will be considered. We will begin working with *S. solfataricus* B12 plasmid SSV1 towards developing a shuttle vector.

**PUBLICATIONS AND REPORTS (last 4 months):** Aspects of this work have been presented at the General Symposium of the (UK) Society for General Microbiology (Edinburgh), Annual Meeting of the American Society of Microbiology (Dallas), University of California (San Diego), Texas A and M University (College Station), the University of Texas (Houston) and the Meeting on Genome Mapping and Sequencing (Cold Spring Harbor).

## ANNUAL PRPGRESS REPORT

GRANT NUMBER N00014-91-J-1141

R&T CODE 4412097---1

PRINCIPAL INVESTIGATOR: Robert P. Gunsalus, Ph.D.

INSTITUTION: University of California, Los Angeles

GRANT TITLE: Genetic Analysis of Hyperthermophilic  
Archaeobacterial Phenomena

REPORTING PERIOD: March 1, 1991 to 15 May, 1991 (two months)

AWARD PERIOD: 1 March, 1991 - 28 February, 1994

OBJECTIVE: To establish and exploit genetic techniques for  
the study of the extremely hyperthermophilic  
archaeobacterium, *Sulfolobus acidocaldarius*.

### ACCOMPLISHMENTS (Last 12 months): (last two months)

Work on the project is only in its second month. Since our laboratory group did not routinely grow hyperthermophiles in the past, we continue to work at establishing optimal culture conditions, including appropriate medium components for cell growth at the desired conditions (75-80 degrees C, pH 5-6), and construction of sufficiently stable incubators. The latter has involved custom modification of a commercially available oven and is still in progress. Most preliminary work has progressed smoothly. Although we anticipate this exploratory phase of our project to go on for several months, essential procedures, such as growth of *Sulfolobus* on defined medium and formation of isolated colonies on plates, have so far been readily achieved in our laboratory. Cells can now be plated at high efficiency (ca 90%) although several variables remain to be identified and controlled in order to obtain this routinely using our standard plating media.

### SIGNIFICANCE:

The study of the hyperthermophilic archaeobacteria, still in its infancy, has proceeded without the aid of classical genetic techniques. In our view, this is a serious limitation which has resulted not from any lack of a suitable organism or of the desirability of such techniques, but of expert effort spent on the problem. Thus, establishing even rather simple procedures such as mutagenesis, isolation of mutant strains, or recovery of genetic recombinants will enable a variety of new studies to be conducted. Examples include analysis of mutational events, manipulation of individual biosynthetic pathways, and development of molecular-genetic tools for the detailed study of dynamic molecular phenomena of hyperthermophilic archaeobacteria.



WORK PLAN (next 12 months):

The objective for the next year is to establish and characterize genetic methods for use with the extremely thermophilic archaebacterium, *Sulfolobus*. We will continue to refine cell plating methods using standard plating media and will work out conditions for use of different media types. We will initiate studies to generate *Sulfolobus* mutants with well-defined phenotypes, including those unable to synthesize amino acids, purines, pyrimidines, or cofactors. Once mutants are obtained, they will be characterized for their reversion frequencies. Pending the availability of suitable mutants, we will initiate studies to examine mutational frequencies of these organisms. We will also attempt to develop methods of genetic transfer for these strains. Finally, as a way to address the broader question of

genetic regulatory mechanisms and responses to environmental stress, we will investigate particular gene products which appear to be regulated.

PUBLICATIONS AND REPORTS (last 12 months): No publications have resulted during the first two months of study on the grant.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-86-K-0739

R & T Code: 441d010

PRINCIPAL INVESTIGATOR: Ramesh Gupta

INSTITUTION: The Board of Trustees of Southern Illinois  
University Carbondale, IL 62901

GRANT TITLE: Structure and Expression of Various RNAs in  
the Archaeobacteria

REPORTING PERIOD: 1 July 1990 - 31 May 1991 (11 months)

AWARD PERIOD: 1 September 1986 -31 July 1991

OBJECTIVE: Characterize the tRNA structure and  
processing in thermophilic archaeobacteria.

ACCOMPLISHMENTS (last 11 months): In our earlier reports we mentioned that we have cloned several tRNA genes of Thermococcus celer. We have sequenced the regions of four of these clones which hybridize to the T. celer total tRNA. Two of the clones contain the same tRNA gene, except that the genes are cloned in two different orientations. These clones have the gene for Asp-tRNA. A third clone contains the gene for Tyr-tRNA. Based on the sequence of the region of the fourth clone, which hybridizes with the tRNAs, a typical clover-leaf structure of the tRNA could not be determined. This clone may contain a pseudogene or the clone may contain only part of a gene which contains a large intron.

Both Asp-tRNA and Tyr-tRNA genes are present as single copies in the genome. These genes do not contain any introns. The 3'-CCA sequence of the mature tRNAs is encoded in these genes. This feature is common for the eubacterial tRNAs but is rare for the archaeobacterial tRNAs. Tyr-tRNA belongs to Class I, i.e., it has a short variable arm, which is a characteristic of archaeobacterial and eukaryotic Tyr-tRNAs. Eubacterial Tyr-tRNAs are of class II type, i.e., contain a large variable arm. By comparing with the consensus archaeobacterial promoter and terminator sequences, putative promoter and terminator regions in these tRNA genes have been identified. Details of the sequences will be provided with the final report of this project.

Limited progress has been made in sequencing of the clone (mentioned in the last report) which potentially contains the two tRNA-methyltransferase genes of Sulfolobus. Several regions of the 9 Kb insert of this clone have been subcloned and some of these subclones have been partially sequenced. So far, we have not been able to find any open reading frame in these sequences.

SIGNIFICANCE: The sequences of T.celer tRNA genes and their flanking regions, as expected, show typical archaebacterial features.

WORK PLAN (remaining period): During the remaining two months of this project, we shall try to confirm the presence of intervening sequence in the "D-loop" region of Sulfolobus tRNA<sup>Glu</sup> gene, which was mentioned in the last report.

PUBLICATIONS AND REPEATS (last 11 months): None

# ANNUAL PROGRESS REPORT

GRANT #: N00014-86-K-0189

R&T CODE: 4413006

PRINCIPAL INVESTIGATOR: Dr. H. Gobind Khorana

INSTITUTION: Massachusetts Institute of Technology

GRANT TITLE: Studies on Biological Light Transduction

REPORTING PERIOD: 1 July 1990 - 30 June 1991

AWARD PERIOD: 1 March 1986 - 1 December 1991

## OBJECTIVE:

- (1) How are the visual photoreceptors constructed? In particular, what are the specific interactive functions of the three domains?
- (2) Mechanism of light-transduction initiated by 11-cis→trans retinal isomerization.
- (3) Studies using retinal analogs that do not allow isomerization upon illumination.
- (4) Continuing study of the interaction of transducin with light-activated rhodopsin.

## ACCOMPLISHMENTS:

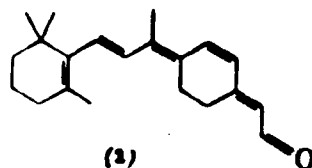
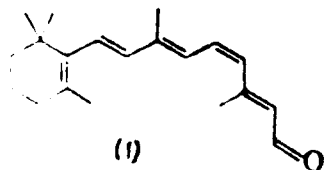
I. Principles on which rhodopsin is built. The membrane-embedded bundle of helices contain the 11-cis retinal chromophore. On one side of the membrane bilayer is the intradiscal face, while the cytoplasmic side faces, on the opposite side, the inside of the cell. This is where all the biochemistry takes place. We have proposed that the intradiscal domain plays a unique structural role and is used to align the 7 helical bundle and probably the cytoplasmic domain.

We are continuing to examine in detail the tertiary structure of the intradiscal domain including a disulfide bond (S-S) between C-110 and C-187, hydrophobic participation at the loops and N-terminus.

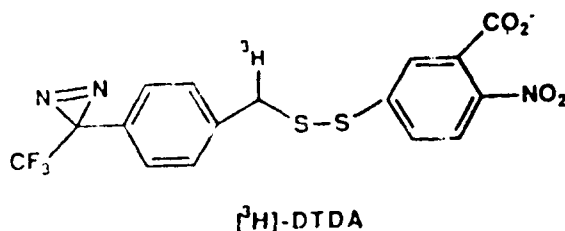
In support of this we found that the deletions, small and large affect the structure and therefore the function.

II. Structure-Function Studies. To understand the conformational changes that occur following activation by light, we have introduced cysteine residues at selective places. Their reactivity in the dark and in the light should indicate the changes in structure. Insights are being gained in regard to the perturbations that the molecule undergoes.

III. Retinal Analogs that do not allow retinal isomerization: For crystallization of rhodopsin and related studies, we have synthesized the two following analogs. The rhodopsin is now much more photoresistant.



IV. Photoaffinity labels for studying interaction between rhodopsin and transducin. The following photoaffinity probe has been synthesized.



SIGNIFICANCE: Our interests are to carry out in depth studies of the biochemical and chemical mechanism of vision. Studies as to how a sensory system, such as vision, works are clearly important and significant. Visual photoreceptors are excellent models for general studies of the mechanism of the superfamily of G protein coupled receptors. Our studies should provide insights into understanding of the mechanism of amplification of signal transduction, adaptation and in brief, how the first event of capture of a photon; namely cis to trans isomerization drives the structural changes.

SELECTED PUBLICATIONS AND REPORTS (last 12 months): (5 of 29)  
 Structure-Function Studies of Bacteriorhodopsin XV: Effects of Deletions in Loops B-C and E-F on Bacteriorhodopsin Chromophore and Structure. M.A. Gilles-Gonzalez, D.M. Engelman and H.G. Khorana (1991) J. Biol. Chem. 266, 8545-8550.

The Reaction of Hydroxylamine with Bacteriorhodopsin Studied Using Mutants that have Altered Photocycles: Selective Reactivity of Different Photointermediates. S. Subramaniam, T. Marti, S. Rosselet, K.J. Rothschild and H.G. Khorana (1991) Proc. Natl. Acad. Sci. USA 88, 2583-2587.

The Role of the Retinylidene Schiff Base Counterion in Rhodopsin in Determining Wavelength Absorption and Schiff pK<sub>a</sub>. T.P. Sakmar, R.R. Franke and H.G. Khorana (1991) Proc. Natl. Acad. Sci. USA 88, 3079-3083.

The Amino Acid Sequence of a Novel Glutamic Acid-rich Protein from Bovine Retina as Deduced from the cDNA Sequence. Y. Sugimoto, K. Yatsunami, M. Tsujimoto, H.G. Khorana and A. Ichikawa (1991) Proc. Natl. Acad. Sci. USA 88, 3116-3119.

Replacement of Leu-93 by Ala or Thr Slows Down the Decay of the N and O Intermediates in the Photocycle of Bacteriorhodopsin: Implications for Proton Uptake and 13-cis\all-trans Retinal Reisomerization. S. Subramaniam, D.A. Greenhalgh, P. Rath, K.J. Rothschild and H.G. Khorana (1991) Proc. Natl. Acad. Sci. USA, in press.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1591

R&T CODE: 441d022

PRINCIPAL INVESTIGATOR: Robert M. Kelly

INSTITUTION: The Johns Hopkins University, Department of  
Chemical Engineering

GRANT TITLE: Hydrogen/Sulphur Autotrophy in the  
Hyperthermophilic Archaeobacterium *Pyrodictium*  
*brockii*

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 31 January 1989 - 1 February 1992

OBJECTIVE: To investigate the bioenergetics of *Pyrodictium brockii* with particular emphasis on the metabolic roles of molecular hydrogen and sulphur. To compare this metabolic mode with heterotrophic or methanogenic metabolisms in organisms growing at similar temperatures.

### ACCOMPLISHMENTS (last 12 months):

#### Sulphur Substrates

Because elemental sulphur serves as a terminal electron acceptor for the growth of *Pyrodictium brockii*, a soluble sulphur substrate is required in order to operate an energy-limited chemostat. As discussed in previous progress reports, various sulphur compounds, including thiosulfate, sulfite, and sulfate were evaluated as possibilities. Unfortunately none of them proved to be suitable for the growth of *Pyrodictium brockii*. Another possibility which was investigated was the use of a polysulfide solution, which we had used before for the growth of *Pyrococcus furiosus*. Although several continuous culture experiments were run successfully using this solution, there was difficulty in operating the culture over its full range of growth rates. It appears that the higher sulfide levels required to solubilize elemental sulphur to polysulfides lead to significant precipitation of essential trace metals, thus limiting the culture. Eventually, a colloidal preparation of elemental sulphur was developed as the sulphur substrate. This preparation has proven useful for the study of other sulphur metabolizing species, as well, e.g. *Pyrococcus furiosus*.

#### Continuous Culture Experiments

Several approaches are being used to gain insight into the energetics of *P. brockii* and *P. furiosus*. An effort is being made to use continuous culture under energy-limiting conditions to estimate growth yields, maintenance requirements, and growth efficiencies for *P. brockii* over its entire temperature range, 80 - 110°C. This coupled with physiological information pertaining to membrane permeability and proton-motive force will be used in developing a mechanistic picture of the overall process. In order to evaluate yield and maintenance coefficients for an organism one must be able to limit the growth of the organism by changing the feed rate of the energy substrate to a chemostat culture. We have had moderate success operating a sulphur-limited chemostat with the colloid, to study the growth of *Pyrodictium brockii*. From these experiments we have been able to estimate a yield coefficient and maintenance rate for *Pyrodictium brockii* growing at 98°C and pH 5.5,  $Y = 0.60$  g cells/mole sulphur and  $m = 0.18$  mole sulphur/g cells hour. However, due to continuing difficulties with cultural stability, we are presently running similar experiments with another organism, *Pyrococcus furiosus*, and plan to get back to *Pyrodictium brockii* after obtaining some data on the energetics of heterotrophs.

*Pyrococcus furiosus* is a heterotrophic hyperthermophile which grows at temperatures from 70 to 103°C and between pH 6 to 8. It is a fermentative organism and will grow on complex substrates such as yeast

extract and tryptone; it will also grow on maltose in the presence of low concentrations of peptides although it can not grow on glucose. It will grow either in the presence or absence of sulphur and although it will produce  $H_2S$  it is not clear what role this sulphur reduction plays in the energetics of the organism. We have recently run a maltose-limited chemostat experiment with *Pyrococcus furiosus* in the absence of sulphur and preliminary estimates of yield and maintenance coefficients are  $Y = 60$  g cells/mole maltose and  $m = 0.01$  mole maltose/g cells hour. In addition to measuring the production of the primary fermentation products,  $H_2$ ,  $CO_2$ , and acetate, we have monitored levels of ammonia,  $\alpha$ -glucosidase and intracellular proteolytic activity in an effort to understand the regulatory pattern of *Pyrococcus furiosus* under these mixotrophic conditions. Of particular interest is the regulation of a novel energetic pathway influenced by the levels of tungsten in the medium.

#### Membrane Physiology Experiments

Some studies in the literature, on more moderate thermophiles, have suggested a correlation between decreasing energetic efficiency and increasing membrane proton permeability as growth temperature is increased. We have developed a protocol for performing membrane permeability measurements on *Pyrodictium Brockii* and obtained half-times, for the decay of an imposed pH gradient, at two different temperatures. These protocols should be useful for making measurements of membrane permeabilities on *Pyrococcus furiosus* with little or no modification. We have built a system that should allow the measurement of permeabilities from room temperature to over  $100^\circ C$ .

**SIGNIFICANCE:** The development of these membrane physiology techniques will allow us to develop better insight into the mechanism behind changes in energetic efficiency. Our culturing system will allow us to quantify the key metabolic parameters of *Pyrodictium Brockii*, *Pyrococcus furiosus* and hopefully other organisms representative of different metabolic modes.

**WORK PLAN (next 12 months):** As mentioned above we have begun a series of experiments on the energetics of *Pyrococcus furiosus* using maltose as the energy-limiting substrate. We plan to determine growth yields, maintenance coefficients and membrane permeabilities for *Pyrodictium Brockii*, *Pyrococcus furiosus* and another heterotroph, ES-4, which requires sulphur for growth and may represent a respiratory heterotrophic metabolism.

#### PUBLICATIONS AND REPORTS (last 12 months):

1. Pihl, T.D., Schicho, R.N., Black, L.K., Schulman, B.A., Maier, R.J., and Kelly, R. M. (1990) "Hydrogen-Sulphur Autotrophy in the Hyperthermophilic Archaeobacterium, *Pyrodictium Brockii*. In: *Biotechnology and Genetic Engineering Reviews* (M.P. Tombs, Ed.), volume 8, chapter 11. Intercept Ltd., Wimborne, Dorset.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-3134

R&T Code:4412070

PRINCIPAL INVESTIGATOR: Hans H. Liao

INSTITUTE: University of Wisconsin Biotechnology Center  
1710 University Avenue  
Madison, WI 53705  
(608) 262-5167

GRANT TITLE: Isolation of Thermostable Enzyme Mutants by Cloning and  
Selection in Thermophilic Bacteria

REPORTING PERIOD: JULY 1, 1990 - JUNE 30, 1991

AWARD PERIOD: JULY 1, 1989 - JUNE 30, 1992

### OBJECTIVES:

- 1) To solve and compare the crystal structures of wildtype and thermostable kanamycin nucleotidyltransferases (KNTases).
- 2) To clone and express KNTase and chloramphenicol acetyltransferase type III (CAT<sub>III</sub>) genes in *Thermus thermophilus*.
- 3) To isolate more thermostable mutants of KNTase and mutants of CAT<sub>III</sub>.

### ACCOMPLISHMENTS (last 12 months):

Objective 1: Analysis of crystals of thermostable KNTase mutant TK101 (Asp80→Tyr and Thr130→Lys) is in progress. The first crystal form (space group C222<sub>1</sub>; unit cell dimensions 128.4 X 156.8 X 155.8 Å) diffracts to <2.4 Å but has 6 enzyme molecules per cell, making solution of the structure complicated. Subsequently, crystals with smaller unit cells (space group P4<sub>3</sub>2<sub>1</sub>2 or P4<sub>1</sub>2<sub>1</sub>2; 78.9 X 78.9 X 220.4 Å) and only 2 molecules per cell were obtained. These crystals were grown in the presence of cobalt ions, suggesting that KNTase binds divalent metal cations; a sequence in KNTase is moderately homologous to Ca<sup>++</sup>-binding sites in other proteins, and Ca<sup>++</sup> inhibits the activity.

Objective 2: Plasmids were constructed to introduce KNTase TK101, the pC194 CAT, CAT<sub>III</sub> (with their native promoters), or TK101 behind the *E. coli* lac promoter into *T. thermophilus*; however, none of these constructs conferred antibiotic resistance to this organism, most likely because the promoters do not function in the thermophile. Selection for resistance yielded only mutants that were not plasmid-associated.

The plasmid carrying KNTase TK101 behind the lac promoter has a unique *Sph*I restriction site in front of this gene for cloning fragments that drive transcription of KNTase in *T. thermophilus*. In an initial test using *T. aquaticus* YT-1 genomic DNA fragments, no plasmid-containing kan<sup>R</sup> colonies were recovered from approximately 3,000 transformants. This experiment is being repeated with more genomic DNA.

A plasmid carrying a *Thermus* promoter that drives KNTase TK101 expression and confers kan<sup>R</sup> was obtained from Y. Koyama (Tsukuba Science City, Japan). M.W. Mather and J.A. Fee (Los Alamos Natl. Lab.) have also reported expression of KNTase in *T. thermophilus* (ASM Meeting, 1991). These results indicate that there are no barriers to the expression of heterologous genes in *Thermus*. The plasmid from Dr. Koyama is now being modified for use as a vector that will express heterologous genes (after mutagenesis by PCR-driven misincorporation) in both *T. thermophilus* and *E. coli* and will permit rapid sequencing of thermostable mutants.



Objective 3: The experiments to generate thermostable mutants of CAT<sub>III</sub> have been frustrated by our inability to make a vector for *in vitro* mutagenesis and transformation into *Bacillus stearothermophilus* due to poor replication in *E. coli* of the pUC18-based plasmid carrying the CAT<sub>III</sub> gene. This construction is being repeated with another parent plasmid.

Other work and new project: A paper on the temperature dependence of expression of KNTase in *E. coli* is in press, reporting that the wildtype protein forms inclusion bodies at 37° but not at 23°C, and that the thermostable mutants were less susceptible to *in vivo* denaturation at 37°C. These results indicate that the mutants have folding intermediates that are more stable, and suggest that elucidation of the folding pathway of KNTase would complement work on solving the crystal structure.

In collaboration with Dr. Kathleen Sandman in Dr. John Reeve's lab (Ohio State University), a new project to isolate thermostable mutants of puromycin acetyltransferase (PAC) as a marker for transformation of thermophilic methanogens is in progress. Dr. Sandman is constructing a vector to express PAC in *B. stearothermophilus*, and I will work on expressing and isolating mutants of PAC in *T. thermophilus*.

SIGNIFICANCE: The set-up phase of this project is largely complete: structural analysis of KNTase is in progress, and the tools for the isolation of more thermostable mutants, by cloning in *Thermus*, are in hand or being constructed. These new mutants will permit further progress on the identification of the critical molecular interactions that confer stability in proteins. In the meantime, the finding that the thermostable mutants are more resistant to denaturation *in vivo* suggests novel methods to avoid the loss of valuable product due to inclusion body formation as well as to study the denaturation process itself.

WORK PLAN (next 12 months):

- 1) Investigate binding and effect of Ca<sup>++</sup> on KNTase by assay and by fluorescence analysis of Tb<sup>++</sup> binding.
- 2) Continue structure determination of KNTase (to be done mostly by collaborators Joshua Sakon and Dr. Ivan Rayment).
- 3) Construct vector for gene expression in *T. thermophilus* and isolate thermostable mutants of KNTase, CAT<sub>III</sub>, and PAC.
- 4) Continue experiments to isolate CAT<sub>III</sub> mutants in *B. stearothermophilus*.
- 5) Initiate fluorescence studies of KNTase structure and folding (with Dr. Catherine Royer, Dept. of Pharmacy, University of Wisconsin-Madison).

PUBLICATIONS (last 12 months):

- Liao, H.H. and Kanikula, A.M. (1990) "Increased efficiency of transformation of *Bacillus stearothermophilus* by a plasmid carrying a thermostable kanamycin resistance marker." *Current Microbiology* 21: 301-306.
- Liao, H.H. (1991) "Effect of temperature on the expression of wild-type and thermostable mutants of kanamycin nucleotidyltransferase in *Escherichia coli*." *Protein Expression and Purification*, in press.

## ANNUAL PROGRESS REPORT

GRANT NO.: N00014-86-K-0222

R & T CODE: 441d006

PRINCIPAL INVESTIGATOR: David P. Nagle, Jr.

CO-PRINCIPAL INVESTIGATORS: David McCarthy and Ralph S. Tanner

INSTITUTION: University of Oklahoma, Norman

GRANT TITLE: Molecular Biology and Physiology of Methanogenic Archaeobacteria

REPORTING PERIOD: 1 July 1990 to 30 June 1991

AWARD PERIOD: 1 July 1988 to 30 June 1991

OBJECTIVES: The long-term objective of this work is to develop a genetic system in the thermophilic methanogen, *Methanobacterium thermoautotrophicum*, to understand its physiology and manipulate it genetically. Specific objectives are to: 1. characterize mutants in purine, pyrimidine, and formate metabolism; 2. isolate mutants with directly selectable phenotypes; 3. generate a simple genetic map; 4. develop a cloning vector for this organism; 5. optimize transformation system; 6. recent objective: define limits to methanogenesis by cathodic depolarization of metals. 7. understand basic metabolism of methanogens and apply this to generation of strains with useful properties.

### ACCOMPLISHMENTS (last 12 months):

-described pathways for purine [1] and pyrimidine [5,6] salvage and interconversion in *M. thermoautotrophicum*; hypoxanthine/guanine phosphoribosyltransferase was purified 200-X; HYP and GUA activation appear to be catalyzed by one enzyme, as in eucaryotic cells [6]. Pyrimidine salvage is by URA phosphoribosyltransferase, nucleoside and deoxynucleoside kinase, and nucleoside but not deoxynucleoside phosphorylase activities (see highlight page) [5,6].

-In *M. thermoautotrophicum* (which cannot synthesize methane from formate) formate is oxidized by a novel formate dehydrogenase which was purified ca. 200-fold [3]. This protein can be used to prepare probes for isolation of the gene which can then be used as a selectable marker in cloning vectors in the formate auxotroph. The role of this enzyme is still unknown.

-Success of transformation experiments had been variable. We found that limiting cells for hydrogen and carbon dioxide (starvation) may lead to competence. With starved recipients in plate- and electro-transformation, fluorouracil resistance and formate prototrophy were transferred reproducibly. This is a result that overcomes previous difficulties [2].

->80% of the *M. thermoautotrophicum* genome was cloned into cosmids. Transposon-mutagenized methanogen DNA was prepared from the cosmid library. This DNA was used to electrotransform wild-type cells to fluorouracil resistance, indicating that the fus gene is in the library. Colony blotting experiments are underway to determine if transposons recombined into the chromosome of the methanogen transformants.

-*M. thermoautotrophicum* synthesized methane with electrons from oxidation of elemental Fe, Mg, Mn, and also from Cu, Ni, and Zn; cat. or concentrations agreed with predictions from methane yields. These results impact on corrosion and biodegradation; notably, Cu, Ni, and Zn ions are toxic to methanogens [4].

**SIGNIFICANCE:** The electrotransformation method streamlines mutant collection and raises the possibility of isolating insertion mutants in any desired gene. Introducing transposon-linked restriction sites will facilitate mapping. The biosynthetic fate of formate remains unknown; the available information indicates that formate will have an anabolic role in other archaebacteria and in anaerobic eubacteria, in addition to methanogens. Methanogens are vital to anaerobic biodegradation processes; we are making steady progress towards our long-range objective to expand the biodegradative capabilities of methanogens.

#### PUBLICATIONS AND PRESENTATIONS (last 12 months):

- A. 1. Worrell, V.E., and D.P. Nagle, Jr. (1990) Genetic and physiological characterization of the purine salvage pathway in the archaebacterium *Methanobacterium thermoautotrophicum* Marburg. J. Bacteriol. 172:3328-3334.
- B. Four abstracts were presented at the General Meeting of the American Society for Microbiology, Dallas, Texas, May 1991.
  2. Bradley, J., and D.P. Nagle, Jr. (1991) Comparison of genetic transformation methods in *Methanobacterium thermoautoautotrophicum*. Abstr. Gen. Mtg. Amer. Soc. Microbiol. H6, p. 156.
  3. Lorowitz, W.H., D.P. Nagle, Jr. and R.S. Tanner. (1991) Formate dehydrogenase from *M. thermoautotrophicum*. *ibid.* I-108, p. 210.
  4. Lorowitz, W.H., and R.S. Tanner. (1991) Methanogenesis from cathodic depolarization of metals by *M. thermoautotrophicum*. *ibid.* I-121, p. 208.
  5. Worrell, V.E., and D.P. Nagle, Jr. (1991) Pyrimidine analog-resistant mutants of *M. thermoautotrophicum* Marburg. *ibid.* I-61, p. 200.
- C. V.E. Worrell was awarded the Ph.D. in Microbiology in May, 1991.
  6. Worrell, V.E. (1991) Purine and pyrimidine salvage pathways of *Methanobacterium thermoautotrophicum* Marburg characterized by genetic and physiological methods. Ph.D. Dissertation, University of Oklahoma, Norman, OK. p. 1-151.

GRANT #: N00014-91-J-1358

R & T CODE: 4412096

PRINCIPAL INVESTIGATOR: Kenneth M. Noll

INSTITUTION: University of Connecticut

GRANT TITLE: Genetic Methods for Rapid Gene Localization in Hyperthermophilic Eubacteria

REPORTING PERIOD: 1 January 1991 - 30 June 1991 (6 months)

AWARD PERIOD: 1 January 1991 - 31 December 1992

OBJECTIVE: To develop genetic tools to investigate the molecular mechanisms of thermophily in and the evolution of the extremely thermophilic eubacterium *Thermotoga neapolitana*.

ACCOMPLISHMENTS (last six months): The efforts of the past six months have primarily been directed toward establishing a new laboratory here to continue the work I began to develop genetic tools for *Thermotoga*. I have begun to work on creating a "top-down" chromosome map of *T. neapolitana* in order to guide our future development of a "bottom-up" map and ordered clone library. I have also initiated a screening for a variety of auxotrophic mutants (including *thy A* mutants). I am also in a position to continue development of a transformation protocol for *Thermotoga*. I have obtained a number of strains of *Thermotoga* which I will screen for plasmids which may be useful for developing a cloning vector.

SIGNIFICANCE: The proposed project is designed to develop the genetic tools necessary to increase our understanding of both the evolution of prokaryotes and thermophilic biological processes. These processes are of interest for both their impact on the marine environment and their potential application to industrial processes. In this project we expect to develop techniques that will allow us to readily identify and clone the genes encoding proteins of interest. *Thermotoga neapolitana* has a number of phenotypic features that are of biotechnological interest. It may be a major source of biogenic sulfide in marine geothermal areas; it grows to high cell densities without producing sulfide, thus simplifying large-scale cultivation; and it is a potential source of thermophilic degradative enzymes (proteases and carbohydrases). As a member of a phylogenetically deep bacterial lineage, it is of special interest for comparative studies with both bacteria and those archaea with which it shares many common physiological features.

WORK PLAN (next 12 months): Four areas of investigation will be pursued in the proposed project. First, auxotrophic and antibiotic-resistant mutants of *Thermotoga neapolitana* will

be isolated for use in genetic analyses. Lac<sup>-</sup> and Thy<sup>-</sup> mutants will be isolated to provide useful selectable markers. In addition, antibiotic-resistance markers that are useful in other thermophiles will be screened to assess their utility in *Thermotoga*. A second project will involve the use of these mutants to refine a transformation protocol that is under development. Preliminary studies indicate that *T. neapolitana* is naturally competent for transformation. A third project will attempt to develop a plasmid cloning vehicle for *Thermotoga* using either an endogenous plasmid or one constructed using portions of cloned *Thermotoga* chromosomal DNA. Finally, a top-down map of the chromosome of *T. neapolitana* will be constructed to guide future mapping and cloning efforts.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-86-K-0211

R&T CODE: 441d003

PRINCIPAL INVESTIGATOR: John N. Reeve

INSTITUTION: The Ohio State University

GRANT TITLE: Molecular Biology of the Extremely Thermophilic Archaeobacteria Methanothermus fervidus

REPORTING PERIOD: 1 July 1990 - 31 May 1991

AWARD PERIOD: 1 April 1988 - 31 May 1992

OBJECTIVE: Our long-term goal is to define, at the molecular level, the mechanisms that enable M. fervidus to grow at temperatures in excess of 80°C. Our current focus is the architecture of the M. fervidus genome, specifically determining how its structural integrity is maintained.

ACCOMPLISHMENTS: We have purified and characterized an abundant DNA-binding protein, designated HMf (histone M. fervidus) from M. fervidus. Previously, [PNAS 87:5788 (1990)] we had shown that this protein binds to dsDNA to produce nucleosome-like structures (NLS). We have now demonstrated that HMf binding to DNA results in an increased number of base pairs (bp) per helical turn and that NLS localization may be directed by intrinsically 'bent' DNA sequences. In the NLS 90-150 bp of DNA are constrained in a positive toroidal supercoil around 4 molecules of HMf. Using the purification scheme developed for HMf we have now also isolated a very similar sized protein (designated HMT), with identical DNA binding properties, from the related thermophilic methanogen Methanobacterium thermo-autotrophicum. We have however been unable to detect such a protein or a cross-hybridizing gene in the related mesophile, Methanobacterium formicicum. The gene hmfB which encodes HMf-2, a subunit of HMf, has been subjected to site-directed mutagenesis. Five variants of rHMf-2 (recombinant HMf-1) have so far been obtained by expression of the mutated hmfB genes in E. coli. Three of these have lost DNA-binding ability, one has reduced DNA binding ability and one displays wild-type properties.

The effects of HMf on transcription in vitro have been investigated in collaboration with Dr. M. Thomm (Univ. of Regensburg, Germany). HMf inhibits transcription of the M. fervidus 7S RNA encoding gene and the hmfB gene but this inhibition can be relieved by adding HMf-free competitor DNA.

An ELISA has been developed to quantitate HMf using affinity purified rabbit anti-HMf antibodies and with the discovery that HMf is soluble in 70% ethanol we can now measure the extent of HMf binding to DNA, very accurately, under a wide range of conditions. Adding ethanol precipitates protein-free DNA molecules and HMf-DNA complexes from mixtures of HMf and DNA. These are then removed by centrifugation and, following evaporation of the ethanolic supernatant, the ELISA can be used to measure the amount of HMf which remained in the supernatant and therefore which did not bind to the DNA. We have used this procedure to investigate HMf binding to DNA at increasing temperatures and in the presence of different concentrations of different salts. The initial results indicate that HMf binding to DNA is dependent on trivalent anions above 60°C.

SIGNIFICANCE: The HMf and HMT based NLS are the only DNA-protein complexes known which contain underwound DNA constrained in positive supercoils. Eucaryal nucleosomes, in contrast, contain overwound DNA (~10.2bp/turn) constrained in negative supercoils. Introduction of positive toroidal supercoils into M. fervidus genome should result in compensatory negative plectonemic supercoiling in protein free regions

of the genome. This may overcome the DNA 'stabilizing' effects of the almost  $1\text{m k}^+$  ion concentration in the cytoplasm of M. fervidus cells which is apparently needed for the functional integrity of its enzymes above  $80^\circ\text{C}$ . The discovery that HMT from M. thermoautotrophicum, a thermophile which lacks reverse gyrase, also produces NLS containing positive toroidal supercoils has eliminated the possibility that this property is needed to compensate for reverse gyrase activity in M. fervidus. The isolation of mutants of hmfB that produce variants of rHMF-2 incapable of binding DNA is the first step towards a detailed structure-function study. Having a protocol that will measure HMF-binding to DNA under a wide range of conditions will allow us to investigate HMF binding to DNA using conditions that mimic, as closely as possible, those in vivo in cells of M. fervidus growing above  $80^\circ\text{C}$ .

**WORK PLAN:** The work in progress to isolate and characterize hmfB mutants will be continued. Structures formed by the interaction of HMF with DNA at high temperatures and in the presence of different salts will be investigated. The gene(s) encoding HMF-1 and HMT will be cloned, sequenced and expressed in E. coli. Complementation of histone deficient yeast mutants and HU-deficient E. coli mutants by expression of the cloned hmfB gene in these cells will be determined.

#### PUBLICATIONS AND REPORTS:

##### Reviewed Articles:

1. Sandman, K., Krzycki, J.A., Dobrinski, B., Lurz, R., and Reeve, J.N. 1990. DNA binding protein HMF, from the hyperthermophilic archaeobacterium Methanothermus fervidus, is most closely related to histone H2A. Proc. Natl. Acad. Sci. U.S.A. 87: 5788-5791.
2. Steigerwald, V.J., Beckler, G.S. and Reeve, J.N. 1990. Conservation of hydrogenase and polyferredoxin structure in the hyperthermophilic archaeobacterium Methanothermus fervidus. J. Bacteriol. 172: 4715-4718.
3. Haas, E.S., Brown, J.W., Daniels, C.J. and Reeve, J.N. 1990. Genes encoding the 7S RNA and the tRNA<sup>Ser</sup> are linked to one of the two rRNA operons in the genome of the extremely thermophilic archaeobacterium Methanothermus fervidus. Gene 90: 51-59.
4. Reeve, J.N. and Beckler, G.S. 1990. Conservation of primary structure in procaryotic hydrogenases. FEMS Microbiol. Rev.
5. Musgrave, D.R., Sandman, K.M. and Reeve, J.N. 1991. DNA binding by the archaeal histone HMF results in positive supercoiling. Proc. Natl. Acad. Sci. U.S.A. Submitted for publication (C. Woese) 5/91.

##### Reviewed Symposium Chapters:

1. Reeve, J.N. and Scherf, B.A. 1990. Methanogenesis and methane genes. Proc. International Symposium on Biotechnology for Energy. Faisalabad, Pakistan ed. Malik, K.A. In press.
2. Krzycki, J.A., Sandman, K.M. and Reeve, J.N. 1991. Purification and characterization of histone HMF from the hyperthermophilic archaeobacterium Methanothermus fervidus. Proc. Sixth International Symp. on the Genetics of Industrial Microorganisms. Strasbourg, France. In press.

##### Reviewed Book Chapters:

1. Palmer, J.R. and Reeve, J.N. 1991. Methanogen genes and the molecular biology of methane biosynthesis. In The Genetics and Molecular Biology of Anaerobes. ed. Sebald, M. In press.

## ANNUAL PROGRESS REPORT

**GRANT #:** N000014-90-J-1823

**R&T CODE:** 441d029

**PRINCIPAL INVESTIGATOR:** Frank T. Robb, Ph.D.

**INSTITUTION:** Center of Marine Biotechnology, University of Maryland, 600 E. Lombard St., Baltimore, MD 21202.

**GRANT TITLE:** Gene Regulation in the Hyperthermophilic Archaeobacterium *Pyrococcus furiosus*.

**REPORTING PERIOD:** July 30, 1990 - May 30, 1991 (10 months)

**AWARD PERIOD:** May 1, 1990 - March 31, 1993

**OBJECTIVE:** To study gene expression and regulation in *Pyrococcus furiosus*, with the goal of obtaining insights into the molecular basis of genetic control mechanisms in hyperthermophiles.

**ACCOMPLISHMENTS:** (last 10 months): We have developed a general probe for serine protease genes using degenerate oligonucleotide primers encoding conserved domains of serine proteases. *P. furiosus* DNA was primed and amplified with these primers, and several amplification products of the appropriate size were detected. One of these products whose internal sequence has homology with subtilisin type proteases is currently being used to screen for genomic clones of *P. furiosus* serine proteases. Antibodies directed against the *P. furiosus* protease S-66 have been used to select clones from the *P. furiosus* cDNA expression library that are currently being sequenced. Several components of the proteolytic apparatus of *P. furiosus* crossreact antigenically with the eukaryotic proteasome, a multienzyme complex including several proteolytic enzymes. The S-66 protease and the proteasome components are derepressed by growth of the cells on limiting nitrogen supply. It is now clear that *P. furiosus* is totally reliant on peptides and amino acids as a nitrogen source, and that the proteases and glutamate dehydrogenase (GDH) occupy pivotal roles in the nitrogen metabolism of this organism. GDH is a major cytoplasmic enzyme that we have purified in large amounts. The amino terminus of GDH has been sequenced and is homologous to yeast GDH. The enzyme has an interesting conformational shift, detected by microcalorimetry, that is analogous to "cold denaturation" of mesophilic enzymes except that it occurs at 72°C. We previously reported obtaining a genomic clone from the *P. furiosus* gene bank that complements an *E. coli* is repeatable, and thermostable "glutamine synthetases" have been purified from extracts of *P. furiosus* and from this clone. We are unable to detect significant homology between the sequence of the cloned DNA and the known glutamine synthetase DNA and amino acid sequences. We believe that the clone expresses a novel enzyme capable of glutamine amidotransferase activity but that it is not a glutamine synthetase in the accepted sense. An archaeobacterial glutamine synthetase probe was deduced from the *Sulfolobus solfataricus* glutamine synthetase sequence. This probe does not hybridize with the putative *P. furiosus* "glutamine synthetase" clone, but it does hybridize with sequences in the genomic DNA of *P. furiosus*. The genomic and cDNA libraries are at present being screened to isolate clones hybridizing with this probe.

Gene regulation has been observed in the case of the promoters controlling the single ribosomal RNA operon of *P. furiosus*. Seven promoters were mapped in the sequenced 1.8 kb region upstream of this operon. The promoters are in two classes with two different consensus sequences: TTAGAAA(-26) and ATATANT(-23). These are the first consensus promoter sequences obtained for a hyperthermophile ( $T_{opt} > 100^{\circ}\text{C}$ ), and they differ from the sequences of *S. solfataricus* promoters (AAANNTTTAAA). We have subcloned this operon into *Haloferax volcanii* using the shuttle vector pWL 102 kindly provided by Ford Doolittle, but we are unable to detect expression of these promoters in a halophile.



**SIGNIFICANCE:** The discovery that the proteases in this hyperthermophile may be organized into a multienzyme complex, containing several catalytic types of protease is another Eukaryote-like features of the Archaea. We have several promising preliminary results regarding cloning of protease genes. The polymerase chain reaction probes for protease and glutamine synthetase are available for studies on the regulation of transcription and for screening the genomic and cDNA libraries. The glutamate dehydrogenase from *P. furiosus* is the most thermostable dehydrogenase reported to date. Multiple, growth rate related rRNA promoters have been mapped and provide the first two consensus sequences for promoters from hyperthermophilic Archaea.

**WORK PLAN:** (next 12 months)

We plan to clone and sequence at least two protease genes from *P. furiosus* and to examine the basis for transcriptional regulation of these genes by nitrogen limitation and growth rate. Using the archaeobacterial glutamine synthetase probe, we will screen the libraries of *P. furiosus* in order to obtain clones with a classical glutamine synthetase sequence. The characterization of glutamine synthetase and glutamate dehydrogenase from *P. furiosus* will continue in order to resolve the roles of these enzymes in primary nitrogen metabolism.

**PUBLICATIONS AND REPORTS:**

Robb, F. T., S. H. Brown and R. M. Kelly. (1990). Molecular Genetic Studies on the hyperthermophilic archaeobacterium. *Pyrococcus furiosus*. Abstracts of the Fifth International Congress on Evolutionary and Systematic Biology. July 1-7, College Park, USA. Workshop Review, In Press.

Fleischmann, E. M., A. R. Place, F. T. Robb and H. J. Schreier. (1991) Protocols for Archaeobacterial Research. University of Maryland Press.

Robb, F. T., J. B. Park and M. W. W. Adams. (1991) Properties of the extremely thermostable glutamate dehydrogenase and glutamine synthetase from *P. furiosus*. Chapter in "Biocatalysis Near and Above 100°C" edit B. C. Tansill, ACS Books. In Press.

## ANNUAL PROGRESS REPORT

GRANT #: N00012-88-K-0464

R&T CODE: 441d018

PRINCIPAL INVESTIGATOR: Kenneth J. Rothschild

INSTITUTION: Boston University

GRANT TITLE: Biophysical Study of Archaeobacterium  
Biomembrane and Possible Application in Biomaterials

REPORTING PERIOD: 1 July 1990 - 30 June 1991

AWARD PERIOD: 1 July 1988 - 30 June 1991

OBJECTIVE: To investigate the molecular mechanisms and basis for structural stability of membrane proteins in Archaeobacteria. An additional objective is to utilize these membranes in the production of new materials which are fabricated on the nanometer scale.

ACCOMPLISHMENTS (last 12 months): Our studies in collaboration with the Khorana laboratory at MIT on the biophysical properties of site-directed mutants of bacteriorhodopsin have led to an increasingly detailed model of the proton transport mechanism (1-8). FTIR, resonance Raman and UV/Vis spectroscopy provide evidence for a retinal isomerization mechanism which involves the residues of Tyr-185 and Asp-212. Evidence was also found for a proton wire which is active in movement of proton during the M to N transition of the photocycle from Asp-96 to the Schiff base. Scanning tunneling microscopy (STM) measurements have been made on the S-layer from the *Sulfolobus Acidocalderis* in collaboration with the N. Clark and K. Douglas at the University of Colorado (9-11). These studies have led to information about the ultrastructure of S-layer and thin metal films patterned on the nanometer level with these membranes.

SIGNIFICANCE: Understanding the molecular basis for function in Archaeobacterial membrane proteins can lead to the development of new types of biomaterials.

WORK PLAN (next 6 months): We have requested a 1/2 year no-cost extension of this project during which time we will bring to completion some of the research discussed in the above section.

### PUBLICATIONS AND REPORTS (last 12 months):

1. Duñach, M., Berkowitz, S., Marti, T., He, Y.W., Subramaniam, S., Khorana, H.G., and Rothschild, K.J. UV-Visible transient spectroscopy of bacteriorhodopsin mutants: Evidence for two forms of Y185F. *J. Biol. Chem.*, **265**, 16978-16984.

2. Rothschild, K.J., Braiman, M.S., He, H.-W., Marti, T., and Khorana, H.G. (1990) Vibrational spectroscopy of bacteriorhodopsin mutants: Evidence for the interaction of aspartic acid 212 with Tyrosine 185 and possible role in the proton pump mechanism. *J. Biol. Chem.* **265**, 16985-16991.
3. Earnest, T.N., Herzfeld, J., and Rothschild, K.J. (1990) Polarized FTIR of Bacteriorhodopsin: Transmembrane  $\alpha$ -helices are resistant to hydrogen/deuterium exchange. *Biophys. J.*, **58**, 1539-1546.
4. Rath, P., Duñach, M., Marti, T., Mogi, T., Khorana, H.G., and Rothschild, K.J. (1990) Raman spectroscopic evidence for a stable m-like form of bacteriorhodopsin mutant Asp-85 to Asn. *Biophys. J.*, **57**, 362a - abstract.
5. Duñach, M., Marti, T., Khorana, H.G., and Rothschild, R.J. (1990) Bacteriorhodopsin mutants of Arg-82, Asp-85, Tyr-185, and Asp-212 do not undergo normal light-dark adaptation. *Proc. Natl. Acad. Sci. USA*, **87**, 9873-9877.
6. Braiman, M.S., and Bousché, O. and Rothschild, K.J. (1991) Submillisecond time resolved FTIR spectroscopy of Bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA*, **88**, 2388-2393.
7. Bousché, O., Raiman, M.S., Marti, T., Khorana, H.G., and Rothschild, K.J. FTIR evidence that Asp-96 deprotonates during the m to n transition of bacteriorhodopsin. *J. Biol. Chem.*, (in press).
8. Subramaniam, S., Marti, T., Rösselet, S.J., Rothschild, K.J., and Khorana, H.G., (1991) *Proc. Natl. Acad. Sci.*, **88**, 2583-2587.
9. Douglas, K., Clark, N.A., and Rothschild, K.J. (1990) Biomolecular/ solid-state nanoheterostructures. *Appl. Phys. Lett.*, **56**, 692-694.
10. Douglas, K., Clark, N.A., and Rothschild, K.J. (1990) Composite biomolecular/solid-state nanostructures. *Mat. Res. Soc. Symp. Proc.*, **174**, 151-156.
11. Douglas, K., Clark, N.A., and Rothschild, K.J. (1990) Biomolecular/ solid-state nanoheterostructures. And, Scanning tunneling microscopy of parallel fabricated nanostructures. *Bull. Am. Phys. Soc.*, **35**, 350.

# ANNUAL PROGRESS REPORT

GRANT#: N00014-89-J-1629

R&T CODE: 441d023

PRINCIPAL INVESTIGATOR: Dr. John L. Spudich

INSTITUTE: Albert Einstein College of Medicine

GRANT TITLE: Role of Protein Methylation in *Halobacterium*  
*halobium* Phototaxis

PERIOD OF PERFORMANCE: 1 July 1990 - 1 July 1991

OBJECTIVE: To investigate the role of methyl-accepting proteins in the phototaxis signaling system of *H. halobium* membranes. A carboxyl-methylated protein in the membrane, MPP-I (methyl-accepting phototaxis protein I) appears to relay the signal from photoactivated sensory rhodopsin I (SR-I, a visual pigment-like photosensor).

PROGRESS (SINCE JULY 1, 1990): (1) Processes important to SR-I activation have been identified; (2) N-terminal sequence has been obtained from MPP-I and cloning efforts initiated; and (3) in collaboration with the laboratory of H.G. Khorana, we have designed and synthesized an SR-I gene optimized for site-directed mutagenesis.

(1) Retinal analog experiments demonstrated that a blue-shifted intermediate, S<sub>373</sub>, is the conformational substate of the protein responsible for producing the attractant signal (Yan and Spudich, 1991, in press). SR-I activation requires steric interaction between the protein and the 13-methyl group on the retinal chain, especially of interest because a similar steric interaction between bovine rhodopsin and the retinal chain 9-methyl group is crucial for photoactivation (in press in PNAS (Yan et al, 1991)). To better monitor the conformational changes of SR-I, we are developing molecular spectroscopic techniques in collaboration with Dr. K.E. Rothschild (Boston University) (in press in Biochemistry (Bousche et al, 1991)).

(2) We have purified MPP-I protein by SDS-gel separation methods and obtained N-terminal sequence, from which we have made 2 oligonucleotide probes for cloning the MPP-I gene. We have also applied high performance liquid chromatography (HPLC), using the system we purchased with the ONR equipment allocation, to isolate fragments of MPP-I which contain the methylation site(s) identified by radiolabeling.

(3) The design and total synthesis of an SR-I apoprotein gene (*srpI*) was accomplished by Mark Krebs, Elena Spudich, and myself while E. Spudich and myself were visiting (Oct - Dec, 1990) the laboratory of H.G. Khorana (Department of Chemistry, MIT). The gene contains unique restriction sites spaced evenly throughout the gene, without changing the amino acid sequence.

SIGNIFICANCE: SR-I provides a model system for understanding receptor activation and signal transduction at the molecular level. The work

combines biophysical analysis with molecular biological tools to elucidate SR-I/MPP-I structure/function.

#### WORK PLAN:

Work over the next year will focus on (1) mutagenesis and expression of the synthetic gene to identify domains and crucial residues of the protein required to activate MPP-I and the methylation system; and (2) the cloning and expression of MPP-I.

The mutated *sopI* will be expressed in *H. halobium* and studied by methods developed in earlier phases of the work on SR-I: (a) *in vivo* signaling properties by automated cell tracking and computer-assisted motion analysis with recent improvements in our laboratory (as in Yan et al 1990b); absorption and photochemistry by UV/visible absorption spectroscopy and laser flash photolysis and (c) *in vivo* modulation of the taxis methylation system (Spudich et al, 1989, PNAS 86:7746-7750). Residues essential to the MPP-I activation will be determined. In parallel, we will use the N-terminal MPP-I probes (or other probes based on sequence in the methylated region of MPP-I) to identify restriction fragments which will then be cloned with a lambda phage cloning system in use in the laboratory.

#### PUBLICATIONS AND REPORTS (last 12 months):

1. Yan, B., Nakanishi, K. and Spudich, J. L. (1991) Mechanism of activation of archaeobacterial sensory rhodopsin-I: Evidence for a steric trigger. Proc. Natl. Acad. Sci. USA, *in press*.
2. Yan, B. and Spudich, J. L. (1991) Evidence the repellent receptor form of sensory rhodopsin I is an attractant signaling state. Photochem. Photobiol. *in press*.
3. Bousche, O., Spudich, E. N., Spudich, J. L., and Rothschild, K. J. (1991) Conformational changes in sensory rhodopsin I: Similarities and differences with bacteriorhodopsin, halorhodopsin, and rhodopsin. Biochemistry *in press*.
4. Yan, B., Takahashi, T., McCain, D. A., Rao, V. J., Nakanishi, K. and Spudich J. L. (1990) Effects of modifications of the retinal beta-ionone ring on archaeobacterial sensory rhodopsin I. Biophysical J. 57: 477-483.
5. Yan, B., Takahashi, T., Johnson, R., Derguini, F., Nakanishi, K. and Spudich, J. L. (1990) All-trans/13-cis isomerization of retinal is required for phototaxis signaling by sensory rhodopsins in *Halobacterium halobium*, Biophysical J. 57: 807-814.
6. Sundberg, S. A., Alam, M., Lebert, M., Spudich, J. L., Oesterhelt, D., and Hazelbauer, G.L. (1990) Characterization of mutants of *Halobacterium halobium* defective in taxis, J. Bacteriology 172: 2328-2335.
7. Takahashi, T., Yan, B., Mazur, P., Derguini, F., Nakanishi, K., and Spudich, J. L. (1990) Color regulation in the archaeobacterial phototaxis receptor phoborhodopsin (sensory rhodopsin II). Biochemistry 29:8467-8474.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1587

R&T CODE: 441d028

PRINCIPAL INVESTIGATOR: Carl R. Woese

INSTITUTION: University of Illinois

GRANT TITLE: Genome Mapping of Thermophilic Procaryotes

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 March 1990 - 28 Feb 1993

OBJECTIVE: To produce a restriction map for the genome of the archaeon Thermococcus celer, which will serve as the basis for a genetic map and for other studies of the genome.

ACCOMPLISHMENTS (last 12 months): At the beginning of this project we ad produced a genomic library for Thermococcus celer, from Sau3A partial digest fragments (15-20 kb inserts) cloned into a lambda phage vector. This was determined from a "top-down" restriction map, assembled from large fragments produced by (three different) rare-cutting restriction endonucleases. The library contained approximately 600 clones, amounting to about 5-6 fold redundancy.

Our strategy has been to split the library into sub-libraries by using the large top down fragments to define them. The first such sub-library, defined by two adjacent SpeI fragments that together total about 200kb, is being investigated in detail, not only to determine the restriction map for this area, but to test accuracy, validity, and speed for the methods used as well. Altogether this 11% of the genome is covered by about 60 clones from the full library. For each clone in the sub-library, the phage is grown and purified, DNA extracted from it, and the DNA characterized by a full digest with four separate hexacutting restriction endonucleases. Relatively rapid methods for preparation of quite pure phage DNA have recently been worked out. [The even more rapid methods used previously proved to give too low yields of DNA of too low a purity to give reproducible results with partial restriction digests, as reported previously]. Given the complete digests (with four enzymes), the sub-library can easily be subdivided into groups of related (overlapping) clones. From each such group an appropriate subset (less than one third of the total in the group) is selected for partial digestion with each of the four restriction endonucleases (the Smith-Bernstiel procedure). From these partial (and complete) digests restriction fragments are ordered in each clone and the clones linked into contigs. In several places the overlap of clones was minimal; in these cases probes were produced from restriction digest fragments of the

clones and the overlap substantiated thereby.

To this point we are just completing the characterization of all clones from the sub-library in question by full digestion with the four restriction endonucleases; about 25 of them have been characterized by the partial digestion method as well; and we have constructed a restriction map for nearly the complete area covered by the two *SpeI* fragments (see above), as shown in the accompanying graph.

Of the 25 clones fully characterized so far (only) one has proven to be a chimera. While this is disturbing (for the library was constructed in such a way that we did not expect chimeras), it was easily detected (by overlap from other clones). This level of artifact should not prove troublesome.

SIGNIFICANCE: To this point the work has served for the most part to develop an accurate procedure for constructing reliable restriction maps for small (prokaryotic) genomes. When the present map is complete, it will serve as the basis for a genetic map and for other studies of the Tc. celer and related genomes. Ultimately the studies will be useful as a guide to sequencing these genomes.

WORK PLAN (next 12 months): During the forthcoming year we will complete the few missing parts of the 11% of the genome discussed above and move on to other sections of the genome. This will involve defining new sub-libraries by means of probes developed from the large top-down fragments. We estimate that pure DNA (in reasonable yield) can be produced at the rate of fifteen clones per person per week. This rate is fast enough to let one person map roughly another 20% of the Tc. celer genome in the forthcoming year. We will also work on making the procedure still more rapid and cost effective.

**MARINE SYMBIOSIS**

ARI (JOINT WITH OCEANIC BIOLOGY)

SCIENTIFIC OFFICER: DR. RANDALL S. ALBERTE

PROGRAM OBJECTIVE: TO IDENTIFY THE BIOCHEMICAL, GENETIC, AND MOLECULAR MECHANISMS THAT GOVERN SYMBIOTIC ASSOCIATIONS OF MARINE ORGANISMS.

NAVY OBJECTIVE: TO CREATE NEW BIOTECHNOLOGICAL CAPABILITIES FOR DEVELOPING NOVEL MATERIALS AND PROCESSES THAT CAN BE USED TO CONTROL BIOFOULING AND BIOCORROSION, AND TO DEVELOP BIOSENSORS, AND BIOREMEDIATION TECHNIQUES.



# ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-3047

R&T CODE: 441h011

PRINCIPAL INVESTIGATOR: Leo W. Buss

INSTITUTION: Yale University

REPORTING PERIOD: 1 July 1990 - 30 June 1991

AWARD PERIOD: 1 July 1989 - 30 June 1992

OBJECTIVE: To determine the transmission dynamics of cnidarian allorecognition using standard breeding experiments and to identify, isolate, and clone the genes controlling allorecognition using molecular methods.

ACCOMPLISHMENTS (last 12 months): We continue our studies of the transmission genetics of allorecognition in *Hydractinia symbiolongicarpus*. In the last 12 months, we have learned that the transitory fusion phenotype, discovered in Year 1, is restricted not only to the onset of reproductive maturity, but that some colonies will exhibit the transitory fusion response long after reproductive maturity is attained. Indeed, all F1 colonies eventually become transitory fusers. Further we have discovered that backcross colonies in the same lineage will exhibit the transitory fusion phenotype even before the onset of reproductive maturity. These results effectively render all prior studies of hydroid allorecognition genetics problematic.

In addition, we have discovered yet another fusibility phenotype in F1 offspring. The phenotype is characterized as follows: a colony initially fuses upon contact, only to show the transitory fusion reaction after a period of 3-4 weeks. Finally after an additional month or more in contact the colony will begin to produce hyperplastic stolons and exhibit what would otherwise appear to be a typical rejection interaction. This is an important finding because it allows us to generate large isogenic colonies differing in fusibility type<sup>1</sup>.

Finally, we have developed methods for the production of cDNA libraries for a colony when exhibiting the transitory fusion interaction and when it is exhibiting the hyperplastic stolon reaction following transitory fusion<sup>2</sup>. It is our intention in the third year of this grant to subtract these libraries in an attempt to identify genes involved in the allorecognition process.

SIGNIFICANCE: Our work to date has demonstrated the existence of no less than three additional allorecognition segregant classes in F1 offspring of *Hydractinia echinata*. These results essentially obviate all prior interpretations of the genetics of cnidarian allorecognition as grossly oversimplified. Moreover and on a more positive note, these new segregant classes offer the potential for use of existing subtractive hybridization strategies for the identification of allorecognition determinants and considerable progress has been made towards realizing this goal.

WORK PLAN (next 12 months): We will continue the analysis of fusibility in our defined genetic lines concentrating on backcrosses and incrosses from our original F1 lineage. It is clear from our work to date that this must be a long range project as some fusibility types do not appear except after as long as one year in culture.

We will subtract the cDNA libraries produced from isogenic individuals displaying different fusibility reactions and subtract the differentially expressed genes from a cDNA library generated from an allogeneic, incompatible colony producing hyperplastic stolons<sup>2</sup>. Genes appearing in the subtracted libraries will be assayed for their co-segregation with fusibility in our defined genetic lines. Differentially

expressed genes will be isolated, cloned and sequenced. From the sequences we will develop RAPD markers to survey our defined genetic lines in order to identify RAPD fragments which co-segregate with fusibility.

SELECTED PUBLICATIONS AND REPORTS (last 12 months):

1. Buss, L.W. and N.W. Blackstone. 1991. An experimental exploration of Waddington's epigenetic landscape. Phil. Trans. Roy. Soc. Lond., in press.
2. Blackstone, N.W. and L.W. Buss. 1991. Shape variation in hydractiniid hydroids. Biol. Bull., in press.
3. Schierwater, B., M. Murtha, M. Dick, F. Ruddle, and L.W. Buss. 1991. Antennapedia class homeoboxes in cnidarians. J. Exp. Zool., in press.
4. Buss, L.W. and M. Dick. 1991. The middle ground of biology; themes at the interface of development and evolution. In: *Growing Points in Evolution*. P.R. Grant and H.H. Horn, eds. Princeton Univ. Press: Princeton. In press.
5. Shenk, M.A. 1991. Allorecognition in the colonial hydroid *Hydractinia* (Cnidarian/Hydrozoa). Amer. Zool., in press.
6. Schierwater, B., W.B. Piekos, and L.W. Buss. 1991. Hydroid stolonial contractions mediated by contractile vacuoles. J. Exp. Biol., in press.

<sup>1</sup> This finding is of technical importance. The ontogenetic change discovered in Yr. 1 allowed us to generate isogeneic colonies of differing fusibility type, hence allowing the development of a subtractive hybridization strategy. However, it was necessary to utilize juvenile colonies to generate one of the two desired libraries and we require gram quantities of tissue to generate the libraries. The new segregant class permits the use of large colonies.

<sup>2</sup> This strategy will obvious lead to the identification of large numbers of genes expressed in the production of hyperplastic stolons. A third library will be generated, using an unrelated, histocompatible colony producing hyperplastic stolons, and this library will be utilized in an additional round of subtraction.

## ANNUAL PROGRESS REPORT

GRANT #: N0014-91-J-1408

R&T CODE: 441t002

PRINCIPAL INVESTIGATOR(S): Clayton B. Cook and Fredric Lipschultz

INSTITUTION: Bermuda Biological Station for Research, Inc.

GRANT TITLE: Development of Regulatory Processes in the Symbiosis Between the Sea Anemone *Aiptasia pallida* and its Dinoflagellate Symbionts

REPORTING PERIOD: 1 March 1991 - 1 June 1991 (3 months)

AWARD PERIOD: 1 March 1991 - 28 Feb 1994

OBJECTIVE: We propose to understand how regulatory processes are initiated and maintained in the symbiosis between dinoflagellate symbionts (zooxanthellae) and the sea anemone, *Aiptasia pallida*. These include the physiological and morphological responses of the algae during the transition from free-living to symbiotic cells, how specific factors in host tissue might elicit these responses, and the nitrogen metabolism of the system.

ACCOMPLISHMENTS (last 3 months): We have begun two phases of this study. In the first, we are developing techniques to infect algae-free anemones with zooxanthellae. Using video techniques with inverted microscopy, we can follow the proliferation of symbionts in individual anemones over time. In the second phase, we are using  $^{15}\text{N}$  (a stable isotope of nitrogen) as a probe to analyze the dynamics of nitrogen metabolism in the normal symbiosis. Our initial studies are aimed at perfecting techniques to isolate host and symbiont samples with minimal contamination, and to identify various nitrogen species in these samples. Our initial results indicate rapid assimilation of ammonium-N by zooxanthellae followed by a slower transfer of this nitrogen to the host.

SIGNIFICANCE: This model system has excellent potential to explore how genomes interact in a symbiotic system. The "host factors" which control symbiont physiology are only expressed when the host is infected with the appropriate symbiont, and the nitrogen metabolism of the intact association is similarly dependent upon the "right" combination of host and symbiont. Our work should clarify how genetic expression by symbionts is influenced by the host environment, and vice versa.

WORK PLAN (next 12 months): Our work plan for the next 12 months will concentrate on three areas. The first is to complete the studies of the infection rates with freshly isolated symbionts, and with cultured symbionts of *A. pallida*. During these studies we will prepare samples of infected tissue for electron microscopy, as a prelude to studies of morphological changes during the course of the infection process. The second area will be to develop assays for putative "host factors" (control of release of photosynthetically fixed carbon, control of amino acid uptake by the algae), and to isolate these factors from host tissue. We plan to collaborate with Dr. R. S. Blanquet of Georgetown University in this work, who has developed HPLC techniques for this purpose. To date he has isolated two active fractions. Our goal is to use immunofluorescent techniques to localize and identify these "factors" in host tissue, as a prelude to studying how the expression of these factors is controlled.

The third area will be the work on nitrogen metabolism. We will refine our techniques and analytical tools to identify and quantify  $^{15}\text{N}$ -species in zooxanthellae and in host tissue. Our next goal is to study how host nutritional history and the density of symbionts in host tissue affects the flux of nitrogen metabolites in the symbiosis, using computer models to analyze these fluxes.

PUBLICATIONS AND REPORTS: An abstract has been submitted to the International Symbiosis Congress (Jerusalem, November 1991).

# FINAL PROGRESS REPORT

GRANT #: N00014-88-K-0440

R&T CODE: 4412045

PRINCIPAL INVESTIGATOR: Aharon Gibor

INSTITUTION: University of California, Santa Barbara

GRANT TITLE: An Amoeba/Zoozanthellae Consortium as a Model System for Animal/Algal Symbiosis

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 June 1988 - 31 May 1991

OBJECTIVE: We have been studying animal/plant symbiosis using a model system of a single cell animal, the amoeba Trichosphaerium, and a unicellular plant, the symbiotic dinoflagellate Symbiodinium.

We have cultured two partners as a consortium as well as independently. We have been describing the life history of the newly isolated and poorly known amoeba, and investigating its interactions with several species of these symbiotic dinoflagellates.

Our major conclusions to date are:

1. The amoebal life history is composed of four distinct morphological forms the prominent one is a multinucleated form ranging in size from about 30-300 microns. This main morphological stage (Morph I) is capable of creating the consortium with the Symbiodinium.
2. The amoebae recognize and differentiate four groups of algae in the genus Symbiodinium. One group of Symbiodinium species were digested by the amoeba with no ill effects to the animal cell. Another Symbiodinium species was toxic when phagocytosed. A third group was barely phagocytosed and mostly avoided, and the fourth group created symbiosis with amoebae which stuffed themselves full of these algae.
3. The amoebae do not only recognize the different algae but also replace less desirable algae already in their cytoplasm with more desirable (digestible and not toxic) species within 15 hours.
4. Two cytoplasmic vacuoles were distinguishable by differential lectin staining in the amoeba. Food vacuoles containing bacteria bound a variety of lectins while the membranes of perialgal vacuoles containing undigested symbiotic algae did not.
5. Like a true symbiont, the algae transferred photosynthetic metabolites to the amoebae and divided inside the cytoplasm.
6. Low maintenance - Long term cultures of the consortium have existed in the culture as a closed system for over four years.
7. The selective digestion by the amoeba enabled the establishment of axenic cultures of the nondigestible algae. Contaminating microorganisms were eliminated by being digested while the intact non-digestible algal cells flourished and were subsequently isolated and cultured axenically.
8. Enzymatic digestion of the algal surfaces by proteolytic enzymes, or coating them with lectins, dramatically changed the uptake rates and, in some cases, the speed of digestion indicating involvement of cell wall surfaces in the initial recognition stage and consortium formation.

## ACCOMPLISHMENTS:

Our major conclusions to date are:

1. The amoebal life history is composed of six distinct morphological forms all of which cycle around a multinucleated stage ranging in size from about 30 to over 200 microns. This main morphological stage is also the one capable of creating the consortium with the Symbiodinium. Two of the transitions between the different forms depends on culture conditions and can be easily induced, these are the transitions between Morph-I and Morph-II and back to Morph-I; and between Morph-I and Morph-III and back to Morph-I. The others occur occasionally in cultures and the conditions for their induction are under investigation.

Trichosphaerium spends most of its life in Morph-I as it grows from 20um up to over 200um in diameter. The cells feed actively and divide about once a day via binary fission. These originally colorless cells engulf Symbiodinium cells and become plump full and deep orange-brown. Such consortia maintained themselves for several years without media change.

2. The amoebae recognize and differentiate four groups of algae in the genus Symbiodinium. One group of Symbiodinium species were digested by the amoeba with no ill effects to the animal cell. They also digest well a large variety of non-symbiotic dinoflagellates such as Gonyaulax, Peridinium, and Gymnodinium. Another Symbiodinium species was toxic when phagocytosed. A third group was barely phagocytosed and mostly avoided, and the fourth group of algae created a consortium filling the cytoplasm of the animal cell. The ability of the amoebae to interact differently with the different algae which supposedly belong to one genus presents a system in which one can study the differences between these algae. Especially the role of surface component(s) in the recognition phenomenon.

3. The amoebae do not only recognize the different algae but also replace less desirable algae in their cytoplasm with more desirable (digestible and not toxic) species within less than 15 hours. The exchange of the perialgal/food vacuole content occurred gradually as amoebae proceeded to engulf newly introduced digestible cells of Chlamydomonas or yeast, and simultaneously released the symbiotic dinoflagellates. The released Symbiodinium was phagocytosed again three weeks later when the digestible algae were consumed and if no other digestible food was made available. In opposite experiments amoebae well fed on Chlamydomonas were exposed to Symbiodinium it became obvious that the exchange of the vacuoles content was controlled. Only minimal numbers of non-digestible algae were taken up by the fed amoebae who ignored the Symbiodinium as long as edible food source was made available (in preparation).

4. Two cytoplasmic vacuoles were distinguishable by differential lectin staining in the amoeba. Food vacuoles containing bacteria bound a variety of lectins while the membranes of perialgal vacuoles containing undigested symbiotic algae did not. The possible existence of two different types of cytoplasmic vacuoles needs to be further characterized.

5. Like a true symbiont, the algae transferred photosynthetic metabolites to the amoebae and divided inside the cytoplasm (Rogerson et al. 1989). The amoebae collected at least 12% of the fixed radioactive bicarbonate, the algae maintained about 60% of the label in them and the rest 28% was lost. The missing radioactivity could have been lost to gaseous CO<sub>2</sub> or to release content of food vacuoles as the cells divided (multiple fission) during the preparation of the consortia for counting (Rogerson et al. 1989).

6. The algae supported low maintenance - long term cultures, of the

present inside and outside of the amoebae. Many algae in the cytoplasm were in various divisional stages, and over 99% of the algae were intact and viable and culturable upon release (in preparation).

7. As a result of the selective digestion, the amoeba were useful for establishing axenic cultures of the nondigestible algal clones. Digestible contaminating microorganisms were eliminated while the intact algal cells were isolated and cultured axenically (Polne-Fuller 1991).

8. Enzymatic digestion of the algal surfaces, or coating them with lectins, dramatically changed the uptake rates and in some cases the speed of digestion indicating involvement of cell surfaces in the initial recognition stage of the consortium formation (Polne-Fuller and Gibor, in preparation).

9. Over 90% of the algae in the amoebal cytoplasm were intact as observed in light and electron microscopy. Over 98% of the algae developed colonies when released from the amoebal cytoplasm. The induction of multiple fission is a useful method for separating between the amoebae and their symbionts, and results in viable populations of algae and amoebae. The morphology of the amoebae releasing their symbionts was transformed from Morph-I into the uniform populations of 20um flat and fan-like Morph-II (Appendix I). Most of these small amoebae were not capable of taking up Symbiodinium cells and starved to death when edible food source was not made available. However, occasionally cells of Morph-II amoebae did engulf algae and survived on Symbiodinium alone creating consortia of one small amoeba containing 1-4 intact algal cells. The biology of the phenomenon of multiple fission and its relations to the amoebal ability to take up algae is under investigation (in preparation).

#### PUBLICATIONS AND REPORTS:

1. Rogerson, A., M. Polne-Fuller, R.K. Trench and A. Gibor 1989. A laboratory-induced association between the marine amoeba Trichosphaerium Am-I-7 and the dinoflagellate Symbiodinium #8. Symbiosis 27:229-241.
2. Rogerson, A., M. Polne-Fuller, and A. Gibor 1991. Lectin binding sites in marine amoebae. Archive. Fur Protistenk (in print).
3. Polne-Fuller, M. 1991. A novel technique for preparations of axenic cultures of Symbiodinium (pyrrophyta) through selective digestion by amoebae. J. Phycol. 27:324-337 (in print).

## ANNUAL PROGRESS REPORT

GRANT N00014-89-J-1742

R&T Code: 4412061

PRINCIPAL INVESTIGATOR: Margo G. Haygood

INSTITUTION: University of California, San Diego  
Scripps Institution of Oceanography

GRANT TITLE: Flashlight Fish Symbiosis

REPORTING PERIOD: 1 August 1990 (last report) - 31 May 1991  
(end of award, 10 months)

AWARD PERIOD: 1 March 1989-31 May 1991

### OBJECTIVES:

1. To investigate the evolution of the symbiosis between luminous bacteria and anomalopid fishes.
2. To test the feasibility of investigating the initiation of the symbiosis in flashlight fish larvae.

### ACCOMPLISHMENTS (last 10 months):

1. We have obtained *luxA* sequence from symbionts of *Anomalops katoptron*, *Photoblepharon palpebratus* and *Photoblepharon steinetzi* and data analysis is underway.
2. Our participation in RRS Discovery 195 was quite successful (see trip report). We have completed sequencing the 16S rRNA genes of the symbionts of the deep-sea anglerfishes *Melanocetus johnsoni* and *Cryptosaras couesi*, and submitted a paper for the Discovery 195 cruise issue of Journal of the Marine Biological Association UK.
3. We have completed sequencing 16S rRNA genes from the symbionts of the anomalopids *Kryptophanaron alfredi*, *A. katoptron*, *P. palpebratus* and *P. steinetzi* and symbiotic *P. phosphoreum* from two opisthoproctids and a macrourid. In collaboration with Reinhardt Rosson, who has sequenced most of the culturable luminous bacteria, we are analyzing the relationships among the group as a whole. Preliminary results were presented at the 1991 General Meeting of the American Society for Microbiology. The preliminary results agree with the RFLP study. The culturable symbionts, even from different families of hosts are identical at the 16S level, while the unculturable symbionts of both the ceratioids and anomalopids are very divergent, although they clearly group with the luminous bacteria. We are now conducting more exhaustive analyses (bootstrapping, etc) in preparation for publication.
4. We have begun a rigorous analysis of copy number of the rRNA operon in the *K. alfredi* symbiont. Our preliminary results show a copy number of two as opposed to the eight found in the facultative symbiont *Photobacterium fischeri*.



### SIGNIFICANCE:

Our genetic work has uncovered evidence of at least six new species of luminous bacteria. We have also shown that the population genetics and evolution of the unculturable (apparently obligate) symbionts is profoundly different from that of the culturable (facultative) symbionts. Symbionts of anomalopids of different genera that occur in the same location do not appear to engage in genetic exchange via seawater populations. The reduced copy number of rRNA operons in the anomalopid symbionts suggests major genetic adaptations to the symbiosis that are not found in the culturable symbionts.

### WORK PLAN:

1. We are attempting to obtain funding from NSF to continue the evolutionary work.
2. When our new award begins we will focus on growth and luminescence regulation in symbiotic and freeliving luminous bacteria.

### INVENTIONS:

None

### PUBLICATIONS AND REPORTS:

- 1990 Haygood, M. G. Relationship of the luminous bacterial symbiont of the Caribbean flashlight fish, *Kryptophanaron alfredi* (family Anomalopidae) to other luminous bacteria based on bacterial luciferase (*luxA*) genes. *Arch. Microbiol.* 154:496-503.
- 1991 Wolfe, C. J. and M. G. Haygood. Restriction fragment polymorphism analysis reveals high levels of genetic divergence among the flashlight fish light organ symbionts. *Biol. Bull.* in press (August 1991 issue).
- Haygood, M.G. D.L. Distel and P.J. Herring. 16S rRNA gene sequences from the luminous bacterial symbionts of the deep sea anglerfishes *Melanocetus johnsoni* and *Cryptosaras couesi*. *Journal of the Marine Biological Association UK*, submitted.
- Haygood, M., R. Rosson and D. Distel. Relationship of the unculturable luminous bacterial symbionts of anomalopid fishes to the culturable marine luminous bacteria determined by 16S rRNA phylogenetic analysis. Annual Meeting of the American Society for Microbiology, Dallas TX, May 5-9 1991

## ANNUAL PROGRESS REPORT

Grant #: N00014-91-J-1357

PRINCIPAL INVESTIGATOR: Margaret McFall-Ngai

INSTITUTION: University of Southern California

GRANT TITLE: Biochemical signaling in recognition and specificity:  
Establishment of a squid/bacterial light organ symbiosis.

REPORTING PERIOD: 1 February 1991 - 31 May 1991 (4 months)

AWARD PERIOD: 1 February 1991 - 31 January 1994

OBJECTIVE: To determine the biochemical and molecular mechanisms underlying host-symbiont recognition and specificity in the mutualistic association between a cephalopod, Euprymna scolopes, and its luminous bacterial symbiont, Vibrio fischeri. Specifically, (1) describe biochemically cell-cell interactions between host and bacteria during the recognition process; (2) identify surface components influencing recognition events; and, (3) analyze the extent to which biochemical recognition processes translate to host range specificity.

ACCOMPLISHMENTS (last 4 months): We focused on four areas during this period -

(1) Visualization of the *in vivo* interactions between host and symbiont: The newly hatched, aposymbiotic host squid has prominent ciliated structures on each of the lateral faces of the incipient light organ. At the base of each of these structures is a series of three pores that lead to spaces colonized by the bacteria during the infection process. Using high-speed cinematography, we have studied the flow field created by these ciliated structures and, with fluorescently labeled bacteria, have studied how these structures entrain bacteria toward the pores of the light organ.

(2) Definition of potential lectins involved in the infection process: We have dissected out newly hatched light organs and tested them for the presence of glycans that have been shown in other systems to be involved in recognition and/or specificity. The symbiotic bacteria have mannose-binding cell surface lectins. The presence of five glycans, the most commonly occurring in animal tissues, were assayed using the Boeringer-Mannheim Glycan Differentiation Kit. Of these only one, a terminal mannose ligand, was detected in the newly hatched light organ, although all five glycans were found in a control tissue (mantle epidermis). Upon infection, the ciliated structures of the light organ are reabsorbed but we determined that the mannose lectins are not lost, indicating that they are probably located within the light organ tubules.

(3) **Characterization of the bacterial cell surface:** Most, if not all, known mechanisms for bacterial infection involve cell surface components of the bacteria. We have begun to define specific cell surface molecules in a strain (ES114) of luminous bacteria isolated from the squid light organ. This strain is being compared to other strains of symbiotic luminous Vibrio fischeri isolated from monocentrid fish light organs; we have identified one such strain (MJ1), which has been in culture for over 12 years, that does not infect juvenile squid, and another (MJ101), which we have newly isolated from monocentrid organs, that is able to infect squid. An antibody to the cell surface of MJ1 (obtained from S. Clegg, U of Iowa) is being used in these comparisons, and antibodies to the cell surfaces of MJ101 and ES114 are being generated.

(4) **Determination of changes in gene expression upon infection:** Significant morphological changes occur in the host in response to the presence of the bacterial symbiont; these changes do not occur when the symbiont has not been introduced. Because such events are likely to be accompanied by specific changes in gene expression, we have begun to characterize the system at the molecular level. We have determined that the newly hatched squid has an average of 30 ng of mRNA per mg of animal tissue. If we assume that the light organ is growing and metabolizing at average rates, calculations of the proportion of the animal volume represented by the light organ has permitted the rough estimate of light organ message as 1/5 of the total mRNA. These estimates, and the isolated mRNA, are permitting the design of experiments to investigate gene expression.

SIGNIFICANCE: The above four approaches to the questions of recognition and specificity have provided background information on these processes in the Euprymna scolopes/Vibrio fischeri association.

WORK PLAN (next 8 months - until the end of the first year of the granting period): We plan to continue to develop the above-mentioned approaches. Specifically -

(1) We have embedded samples for frozen sectioning, which we will use to determine the exact location of host glycans. Further, we will attempt to inhibit infection by incubation of the bacteria with mannose before infection to block mannose-sensitive sites and incubation of the animal with lectins that will mask mannose-sensitive sites.

(2) Using antibodies to the bacterial cell surfaces of the above-described strains, we hope to define cell surface components specific to the fully virulent ES114 strain. The antibodies will be used in western blots of the cell surface proteins, and for subtractive precipitation of shared components among the different strains.

(3) By PCR-based differential screening [Science (1991) 252:856], we will study differences in gene expression between aposymbiotic and symbiotic juveniles. Thus, we will determine the influence of the presence of the bacteria on the host at the level of the genome.

PUBLICATIONS AND REPORTS (last 4 months): none.

# ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-4005

R&T CODE: 4412027

PRINCIPAL INVESTIGATOR: Daniel E. Morse

INSTITUTION: Marine Biotechnology Center  
University of California, Santa Barbara

GRANT TITLE: Molecular Marine Symbiosis

REPORTING PERIOD: 1 August 1990 - 30 May 1991

AWARD PERIOD: 1 August 1990 - 31 July 1991

OBJECTIVE: To characterize the molecular mechanisms controlling substratum recognition, adhesion and metamorphosis of the larvae of model macrofouling organisms on surfaces immersed in the ocean.

ACCOMPLISHMENTS (last 12 months): We have continued our *in vitro* dissection of the molecular mechanisms controlling settlement and metamorphosis in larvae of the mollusc, *Haliotis rufescens*, and have further characterized the molecular adhesive and the mechanisms controlling fouling by the cementing polychaete, *Phragmatopoma californica* (Jensen, 1991). Cilia purified from the *Haliotis* larvae contained the chemosensory receptors and signal transducers controlling substratum-specific attachment and metamorphosis in response to two classes of chemical signals from the environment: a peptide signal associated with the recruiting services, and an amino acid present in variable concentrations in seawater. The (lysine) amino acid receptor and its signal transducing cascade of receptor-regulated G protein, phospholipase C and protein kinase C were resolved and analyzed *in vitro*. Reciprocal control of the chemosensory receptor by its G protein suggests that the receptor may be a transmembrane protein of the rhodopsin superfamily. The purified chemosensory cilia were found to contain mRNA encoding elements of the chemosensory pathway. Synthesis, PCR-amplification, and sequence analysis of the corresponding cDNAs led to identification of two G protein mRNAs in the chemosensory cilia. One of these is closely homologous to the Gq family recently found in mammalian brain, and also shown to regulate phospholipase C. This pathway in the larvae amplifies the settlement response to the surface-associated inducers. Our nucleic acids studies in the larvae were facilitated by our development of a new technique for purification of full-length larval cDNAs. These studies also demonstrated that larval metamorphosis induces the expression of a potent digestive serine protease; cDNA sequence analyses and Northern hybridizations show that this enzyme is a unique ancestral chymotrypsin, highly and specifically expressed in the newly differentiated cells of the distal intestine.

We have shown that the adhesive protein secreted as a tube cement by *Phragmatopoma* is the chemical inducer of gregarious larval settlement of this macrofouler. In collaboration with Professor H. Waite (U. DE) we have accomplished the biochemical purification and sequence analyses of the principal DOPA-containing peptides of this adhesive protein precursor molecule (Waite, Jensen and Morse, in prep.). We have shown that this protein, and a chemical analog of its DOPA crosslinks, are potent inducers of substratum specific larval settlement both in the laboratory and in the natural ocean environment (Jensen and Morse, 1990). We have also shown that fatty acids are not present in the natural inducer; although these compounds can be obtained as contaminants of the inducer artifactually, they induce larval settlement non-specifically. We recently have obtained evidence for a calcium-dependent control of metamorphosis in *Phragmatopoma*, possibly involving

protein kinase C.

**SIGNIFICANCE:** Our results demonstrate that purified larval cilia provide a model system uniquely suited for *in vitro* resolution of the chemosensory receptors and signal transducers controlling metamorphosis in planktonic marine invertebrate larvae. Our discovery that functional mRNA can be purified from these cilia in quantities sufficient to establish a cDNA library extends the tractability of the *Haliotis* larval system to analyses of the chemosensory elements of the cDNA and protein sequence level. This is the first discovery of functional mRNA in cilia from any system, and itself is likely to open new areas of research in many laboratories. Results of these studies are helping to elucidate the detailed molecular mechanisms by which chemosensory receptors and transducers regulate larval settlement behavior and metamorphosis. Results of the continuing cDNA investigations should provide insights into the basic mechanisms of action (and the evolution) of the chemoreceptors and their associated signal-transducers in the molluscan larvae. The mechanisms by which these control responsiveness to stimuli, physiological and behavioral processes, and the activation of gene expression and development, can be expected to be applicable to a wide variety of other sensory, neuronal, hormonal and developmental systems as well. These results may help us to identify new targets and strategies for the prevention of larval settlement attachment and biofouling through non-polluting means. The structural characterization of the adhesive protein from *Phragmatopoma* may lead to the development of useful new composite biomaterials, including underwater and medically useful adhesives.

**WORK PLAN (final 2 months):** We aim to conclude the studies of the adhesive protein, and complete publication of the work described above.

**SELECTED PUBLICATIONS AND REPORTS (4 of 11):**

1. Morse, D.E. 1990. Recent progress in larval settlement and metamorphosis: Closing the gaps between molecular biology and ecology. *Bull. Mar. Sci.* 46:465-483.
2. Jensen, R.A. and D.E. Morse. 1990. Chemically induced metamorphosis of polychaete larvae in both the laboratory and ocean environment. *J. Chem. Ecol.* 16:911-930.
3. Jensen, R.A., D.E. Morse, R.L. Petty and N. Hooker. 1990. Artificial induction of larval metamorphosis by free fatty acids. *J. Exp. Mar. Biol. Ecol.* 67:55-71.
4. Wodicka, L. and D.E. Morse. 1991. cDNA sequences reveal mRNAs for two G $\alpha$  signal transducing proteins from larval cilia. *Biol. Bull.* 180:318-327.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-3246

R&T CODE: 4412074

PRINCIPAL INVESTIGATOR: Leonard Muscatine

INSTITUTION: Department of Biology, UCLA

GRANT TITLE: Mechanism by which cold shock evokes exocytosis of symbiotic algae in marine cnidarians.

REPORTING PERIOD: 1 July 1990 - 30 May 1991

AWARD PERIOD: 30 September 1989 - 30 December 1992

OBJECTIVE: To determine the mechanism by which low temperature evokes release of symbiotic algae from marine cnidarians.

ACCOMPLISHMENTS (last 11 months): We have discovered that release of zooxanthellae from marine cnidarians is not by exocytosis, but by release of whole, intact cells. (The host cells disintegrate after a few hours and give the impression thereafter that naked algae are released). Release of whole cells is the result of cell adhesion dysfunction. This form of release is now supported by 1) direct observation with epifluorescence microscopy. The animal cell cytoplasm can be visualized with fluorescein diacetate, and the nucleus by the Hoechst fluorochrome; 2) direct observation of the product of release by SEM and TEM; 3) detection of host cell protein released in direct proportion to release of zooxanthellae.

This finding allows us to put forward the following testable working hypothesis: Low temperature causes membrane phase changes which permit passive influx of calcium ions into host cells. The elevated calcium leads to collapse of elements of the host cytoskeleton. Instability of actin, microtubules, vimentin, and cytokeratin at high calcium concentrations is well known. The effect of collapse of the cytoskeleton is also known to cause dysfunction of cell adhesion molecules, particularly the calcium-dependent cadherins.

We tested this hypothesis by attempting to evoke release of zooxanthellae at room temperature using pharmacological agents which promote calcium influx. We obtained equivocal results with A23187 ionophore, and verapamil, but obtained positive results with BAY K 8644, a calcium channel agonist, high external calcium, and high external potassium. We also obtained positive results by electroporation of anemones at 1100 volts in sea water. The effect of BAY K could be reversed with nimodipine, a calcium channel antagonist.

Our second major accomplishment was the development of two methods for macerating anemone tissue to release whole, living cells. We employ collagenase or calcium-free sea water, depending on the cnidarian. We use flow cytometry to determine the number of algae per cell and the frequency distribution of each cell type in the population of cells. We can now investigate the behavior of intact, isolated living host cells at low temperature.

We chilled isolated cells in the presence of cell-permeant calcium-dependent fluorochromes and, using computer-enhanced image analysis, observed change in cell shape, blebbing and elevated intracellular calcium at low temperatures. This observation is consistent with our working hypothesis. We are currently attempting to employ direct and indirect immunofluorescence to determine the status of the various cytoskeletal elements during cold shock. We are also developing an electronic thermal microscope stage so that we may alter temperature more precisely and observe the subsequent behavior of living isolated cells. We also applied heat shock to a common Hawaiian reef coral and observed the same result - release of whole, intact cells. If this turns out to be a general result, it has considerable significance

for the problem of coral bleaching, as it now defines a specific cellular response to elevated temperature and implicates adhesion dysfunction.

We have experimental evidence showing that host cells can adapt to low temperature. Adapted cells give a much lower response to cold shock. We are investigating the mechanism of adaptation by attempting to determine if membrane fatty acids are altered during acclimation to low temperature.

SIGNIFICANCE: The release of intact cells containing zooxanthellae cold shock is significant in the following ways. It permits us to focus on the mechanism - cell adhesion dysfunction. In addition, acute and chronic "bleaching" by corals and other tropical cnidarians has been observed for more than a decade. There is increasing evidence that loss of photosynthetic pigment and release of zooxanthellae from tropical cnidarians is coincident with elevated sea water temperatures. The elevated temperatures are thought by some to be part of a general trend in global warming. In any case, elevated (and low ) temperature will evoke release of zooxanthellae (Hoegh-Guldberg, 1989; Glynn and D'Croz, 1990). However, in spite of numerous research investigations on coral bleaching, virtually nothing is known of the cellular level events which accompany destabilization of these symbioses. Our findings describe the form of release of zooxanthellae and establish the hypothetical mechanism by which elevated temperature evokes the release response. The immunofluorescence bioassay for altered cytoskeletal elements may be useful in detecting heat stress in marine organisms.

WORK PLAN: We will attempt to describe the disposition of key elements of the cytoskeleton (initially microtubule, actin filaments, vimentin and cytokeratin intermediate filaments) and cell surface adhesion molecules (initially cadherins) using indirect immunofluorescence bases of fluorochrome-conjugated antibodies specific for each of the proteins of interest. We shall also attempt to evoke cell detachment at normal temperature with inhibitors of microtubule assembly and disassembly (colchicine, vinblastine, taxol); inhibitors of actin function (cytochalasin B, phalloidin, phorbol esters); and intermediate filament disrupters (acrylamide).

A selected cadherin, probably E-cadherin, will be chosen for analysis initially. We shall attempt to detect cadherin synthesis and location by immunofluorescence using fluorochrome conjugated antibodies to the target protein. We shall then determine if the abundance and distribution of the protein changes is altered by low temperature. We shall also be interested to determine if E-cadherin can be co-localized with actin filament bundles. We shall compare results in control cells released by maceration with those released by cold shock. We shall cold shock the intact anemone and then examine host cells after tissue maceration (in vivo stress). We shall also macerate tissue to obtain living cells and then cold shock them on a temperature-controlled microscope stage and observe them during and after the stress treatment (in vitro stress) by application of the immunofluorescence assay. Finally, we will analyze membrane fatty acids in control and cold-acclimated anemones by gas chromatography.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-91-J-1359

R&T CODE: 441t004

PRINCIPAL INVESTIGATOR: R.L. Pardy

INSTITUTION: University of Nebraska, Lincoln

GRANT TITLE: Biochemical Analysis of Perialgal Membranes, Cell Walls and Plasma Membranes of Symbiotic Algae.

REPORTING PERIOD: 1 March 1991 - 31 May 1991 (3 months)

AWARD PERIOD: 1 March 1991 - 28 February 1994

OBJECTIVE: 1) To analyze the biochemical and dynamical properties of symbiotic algal cell-walls, plasma membranes, and the perialgal membrane, and 2) to study the comparative genetic regulation of the interface. From these studies we hope to discover those molecular properties unique and significant to the establishment, maintenance and evolution of the symbiosis.

ACCOMPLISHMENTS (last 3 months): Our accomplishments are as follows:

- 1) setup and calibration of HPLC equipment,
- 2) establishment of symbiotic cell cultures,
- 3) preparation of immunoglobins to algae (in progress),
- 4) refinement of cell-wall isolation procedures, and
- 5) analysis of cell-walls for mono- and di-saccharides.

SIGNIFICANCE: We are now preparing composition profiles of cell-walls of various species and strains of symbiotic and free-living algae. These profiles will be with special regard to mono- and di-saccharides, amine sugars, amino acids, peptides and proteins, and glycoproteins. We believe development of these proteins early in our research will expedite the differentiation of the various algal types. Furthermore, we hope our analyses will lead us to basic biochemical information important for the determination of cell-wall constituents unique to algae in symbiosis.

WORK PLAN (next 9 month): The objective for the rest of the year is to complete the cell-wall composition profiles, to make comparative analyses among the various strains and between symbiotic and free-living types, and to begin work on identification of those molecules unique and/or significant to the symbiosis. It is our intention to include HPLC and immunochemical techniques to facilitate these analyses and characterizations.

PUBLICATIONS AND REPORTS: Presently there are no publications resulting from work supported by this grant.



## ANNUAL PROGRESS REPORT

GRANT #: N00014-88-K0463

R&T PROJECT CODE: 4412049

PRINCIPAL INVESTIGATOR: Robert K. Trench

INSTITUTION: University of California, Santa Barbara

GRANT TITLE: The Genetic Basis of Specificity in Dinoflagellate- Invertebrate Symbiosis

PERIOD OF PERFORMANCE: 1 July 1990 - 30 June 1991

AWARD PERIOD: 1 July 1988 - 30 June 1991

OBJECTIVE: (1) To use the antibodies prepared against the exuded glycoproteins from *S. pilosum* (an alga that does not infect *Cassiopeia*) and *S. kawagutii* and *S. pulchrum* (two algae that do infect *Cassiopeia*). (2) Use the antibodies in immunocytochemical assays to detect the glycoproteins in hospite. (3) Attempt in vitro analyses of binding of glycoproteins to isolated symbiosome membranes.

With regards to the phylogeny of symbiotic dinoflagellates, we hoped to complete sequencing the 17S rDNA from three symbiotic and one non-symbiotic dinoflagellates.

ACCOMPLISHMENTS (LAST 12 MONTHS): As described in our last report, the exuded glycoproteins produced two distinct components on Sephadex G-100 chromatography. We had polyclonal antibodies prepared against the large molecular weight fraction from *S. microadriaticum* (anti-SmxuL). These antibodies reacted strongly in immunoblot analyses with the large molecular weight fraction after separation by SDS electrophoresis. No cross-reactivity was observed with the small molecular weight fraction.

The anti-SmxuL antibodies were reacted in immunoblot assays with SDS-PAGE-separated total exudate from *S. kawagutii*, *S. pilosum*, and *S. pulchrum*. No cross-reactivity was detected in *S. kawagutii* or *S. pulchrum*. A large molecular weight (about 250 kDa) component of the exudate from *S. pilosum* did cross-react.

Since *S. pulchrum* and *S. kawagutii* infect *C. xamachana* and *S. pilosum* does not, it is reasonable to conclude that the component of the exudate of *S. pilosum* that is immunologically similar to exudate from *S. microadriaticum* is not involved in recognition. It also raises the possibility that the large molecular weight component of the exudate may not be involved in the recognition process. We have now had polyclonal antibodies prepared against the small molecular weight fraction of exudate from *S. microadriaticum* (anti-SmxuS antibodies), which demonstrate high specificity for the small molecular weight fraction in immunoblot assays after SDS-PAGE separation, and we will use the antibodies to repeat the experiments described above.

We have conducted immunocytochemical assays at the light microscope level using scyphistomae of *C. xamachana* infected with *S. microadriaticum* and anti-SmxuL antibodies. FITC-conjugated goat-anti-rabbit antibodies were used as the secondary antibodies. The results indicated that there is movement of exudate from the algae to animals in hospite. The fluorescent "tag" could be detected in association with certain animal cell mucus glands, and at the base of the oral disc of the scyphistomae, with the nuclei of certain cell types. This latter observation produced a great deal of excitement, in that it suggests that some component of the exudate may have DNA-binding capability, and could be the signal from the alga to the animal that initiates the developmental sequence leading to strobilation, which only occurs after infection of the scyphistomae with an appropriate symbiont.

We intend to corroborate this finding in the following ways: (1) the experiment will be repeated with assays at the light microscope level using colloidal gold-conjugated secondary antibodies, followed by enhancement with silver (a technique that we have only recently become familiar with, and which obviates problems with antifuorescence when using FITC); (2) at the E.M.

level with gold-conjugated secondary antibodies (which should provide more detailed localization); and (3) having isolated DNA from the scyphistomae, conduct gel retardation experiments using <sup>32</sup>P-DNA from the scyphistomae and exudate from the algae. This should indicate whether there is some component of the exudate from the algae that does not bind to animal DNA.

We have not yet attempted to isolate symbiosome membranes and conduct ligand-receptor binding experiments with the exudate.

To determine if there are components of the algal exudate that are chemically similar to that of the scyphistomae that may be involved in the animal's inability to distinguish self from non-self, we extracted total protein from scyphistomae, separated them by SDS-PAGE, and conducted immunoblot assays using anti-SmxuL antibodies. No cross-reactivity was detected. We will repeat this assay with the anti-SmxuS antibodies.

Summary: The complete small ribosomal subunit RNA (17S rRNA) sequence was determined for the symbiotic dinoflagellate *Symbiodinium pilosum*. This sequence was compared with sequences of 2 other dinoflagellates (*Prorocentrum micans* and *Cryptothecodinium cohnii*), 5 Apicomplexa, 4 Ciliata, 5 other eukaryotes and one archaeobacterium (used as an outgroup). By two analytical approaches, the dinoflagellates appear distantly related to prokaryotes, and are most closely related to two of the Apicomplexa, *Sarcocystis muris* and *Theileria annulata*. Among the dinoflagellates, *C. cohnii* was found to be more closely affiliated with the Apicomplexa than either *P. micans* or *S. pilosum*.

**SIGNIFICANCE:** The finding that the symbiotic algae do release glycoproteins *in hospite* is significant on two counts (1) some component(s) of these glycoproteins could be signals passing between symbiont and host and (2) in addition to indicating the propriety of a given alga, might also be involved in the activation of developmentally important genes. When the amino acid composition of the glycoproteins is taken into consideration, it could also explain the algal source of several essential amino acids, not previously accounted for.

**WORK PLAN (next 12 months):** Confirm observations on glycoprotein release, and binding to host DNA by light microscope and EM immunocytochemistry. Repeat the experiments described above with the anti-SmxuS antibodies. Conduct gel retardation experiments using isolated and purified DNA from scyphistomae and exudate from *S. microadriaticum*.

**PUBLICATIONS AND REPORTS:** Govind, N.S., Roman, S.J., Iglesias-Prieto, R., Trench, R.K., Triplett, E.L., and Prezelin, B.B. 1990, An analysis of the light-harvesting peridinin-chlorophyll a-proteins from dinoflagellates by immunoblotting techniques. *Proc. R. Soc. Lond. B.* 240: 178-195.

Matta, J.L. and Trench, R.K. 1991, The enzymatic response of the symbiotic dinoflagellate *Symbiodinium microadriaticum* (Freudenthal) to growth *in vitro* under varied oxygen tensions. *Symbiosis* 11 (in press).

Markell, D.A., Trench, R.K. and Iglesias-Prieto, R. 1991, Macromolecules associated with cell walls of symbiotic dinoflagellates. *Symbiosis* 12: (in press)

Trench, R.K. 1991, Current trends in the study of microalgal-invertebrate symbiosis. In: *Encyclopedia of Microbiology*.

J. Lederberg (ed.) Academic Press, New York. (in press).

## **MARINE VIRUSES**

### **CORE PROGRAM**

**SCIENTIFIC OFFICER: DR. ERIC EISENSTADT**

**PROGRAM OBJECTIVE: TO DETERMINE THE FUNDAMENTAL MECHANISMS OF INTERACTION BETWEEN MARINE VIRUSES AND THEIR NATURAL MARINE HOSTS.**

**NAVY OBJECTIVE: TO DEVELOP TOOLS FOR DEVELOPING NEW BIOTECHNOLOGIES WITH MARINE ORGANISMS THAT WILL PROVIDE NOVEL MATERIALS AND PROCESSES FOR ADDRESSING SUCH NAVAL NEEDS AS THE CONTROL OF BIOFOULING OR THE DEVELOPMENT OF NEW TREATMENTS FOR ELIMINATING HAZARDOUS WASTE IN MARINE ENVIRONMENTS.**

# ANNUAL PROGRESS REPORT

GRANT#: N00014-90-J-1693

R&T CODE:4412076---01

PRINCIPAL INVESTIGATOR: Dr. Russel H. Meints

INSTITUTE: Oregon State University

GRANT TITLE: Molecular Investigation of a Virus Infecting a Eukaryotic Marine Alga

REPORTING PERIOD: 1 July 1990- 1 July 1992

OBJECTIVE: To carry out the first molecular characterization of a virus infecting a eukaryotic marine alga, and to determine whether this alga-virus system can be used as an experimental genetic tool for the study of the molecular genetics of brown algae (Phaeophyceae).

ACCOMPLISHMENTS (last 12 months): We have succeeded in purifying particles of the virus (Fsv) on sucrose gradients, and we have used the purified virions to analyse the viral proteins by SDS-PAGE, and for analysis of viral DNA by CHEF (Clamped Homogeneous Electric Fields) pulsed-field electrophoresis. We have resolved the viral DNA as two genomes of 182 and 165 kbp. The two DNA's are equally abundant in virions from cultures grown at 20°C, but at 10°C the smaller is considerably less abundant. Clonal algal isolates established from single vegetative cells of the infected alga also yield infections possessing both bands. Hybridization to Southern blots of the two DNA's with probes of total-virus DNA as well as with small (ca. 10kb) clones from our BamHI library of Fsv DNA revealed that they may be similar (or essentially identical), the differences being the reduced size and loss and/or gain of restriction sites. We surmise that the virus may exist in two forms, one being a temperature-sensitive deletion mutant.

A Fsv gene transcript of ca. 1200 bases has been identified by hybridization of a BamHI clone to Northern blots of RNA extracted from the virus-infected alga. The open reading frame is being characterized. A probe made from a gene coding for a 33kd protein in the Chlorella-virus PBCV-1 has shown a strong hybridization signal on Northern blots.

We have collected throughout the year and established in culture a second virus-infected brown alga, from several localities on the Oregon coast (our Fsv isolate is from New Zealand). We have also established cultures of uninfected virus host.

We have tested the sensitivity of the host to a sulfonylurea herbicide in order to determine a selectable marker for a transformation system. The alga is sensitive to Glean at ~2-5 µg/ml.

SIGNIFICANCE: This first molecular characterization of a marine algal virus, and first investigation of the host range of a marine algal virus should provide important basic information about these poorly known marine pathogens. Development of a genetic transformation system for marine algae would greatly enhance the potential of these organisms for production of biomass energy and biopolymers through biotechnology.

WORK PLAN (next 12 months): We will complete sequence analysis for the isolated gene, in particular comparing promotor regions with those of Chlorella-viruses. Transcriptional analysis is in progress. We will continue efforts to identify features (besides size) of the two Fsv

DNA's that distinguish between them, to determine the significance of the occurrence of two genomes in what is ostensibly one virus infection. We are preparing biotinylated FSV DNA probes for fluorescent *in situ* hybridization studies of the localization of viral DNA and RNA in the cells of algal hosts. This should provide direct evidence of integration and the site of virus replication.

The nuclear gene for acetolactate synthase will be isolated by PCR technology and characterized. *In situ* mutagenesis will be used to produce a "resistance" gene to be used in transformation studies. We will attempt to prepare recombinant virus bearing the GUS gene under control of the virus promoter from the gene described above.

We will continue to study the virus from the Oregon coast. We hope to carry out infection studies and assess geographical variation, which has not been possible with FSV, which is presently known only from one site in New Zealand and lacks an uninfected host. We also plan a collecting trip to New Zealand to obtain FSV from multiple sites and to search for uninfected hosts.

#### PUBLICATIONS AND REPORTS (last 12 months):

Three abstracts submitted for presentation at the Fourth International Phycological Congress, Duke Univ., August 4-10, 1991 (copies enclosed):

Graves, M. V. and R. H. Meints (1991). Cloning of the Gene for VP54, the Major Capsid Protein of *Chlorella* virus PBCV-1. Abstract.

Henry, E. C., S. K. Krueger and R. H. Meints (1991). Analysis of the genome of a virus infecting a brown alga. Abstract.

Meints, R.H. (1991). Viruses of exsymbiotic *Chlorella*: Comparison between viral and host nuclear encoded genes. Abstract.

#### Other work:

Amberg, S.M. and R.H. Meints. (1991). Nucleotide sequence of two chloroplast genes from a *Chlorella*-like green alga: the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase and ribosomal protein S14 (J. Phycol., in press)

Schuster, A.M., J.A. Waddle, K. Korth and R. H. Meints. (1990). The chloroplast genome of an exsymbiotic *Chlorella*-like green alga. Plant Mol. Biol. 14:859-862. (copy enclosed)

Schuster, A.M., M.V. Graves, K. Korth, M. Ziegelbien, J. Brumbaugh, D. Grone and R.H. Meints. (1990). Transcription and sequence of a 4.3-kbp fragment from a ds-DNA eukaryotic algal virus. Virology 176:515-523. (copy enclosed)

Van Etten, J.L., L. Lane and R.H. Meints (1991). Viruses and virus-like particles of eukaryotic algae. Microbiological Reviews. (in press). (copy enclosed)

Waddle, J.A., A.M. Schuster, K.W. Lee and R.H. Meints. (1990). The mitochondrial genome of an exsymbiotic *Chlorella*-like green alga. Plant Mol. Biol. 14:187-195. (copy enclosed)

## ANNUAL PROGRESS REPORT

GRANT#: N00014-90-J-1280

R&T CODE: 423a016---01

PRINCIPAL INVESTIGATOR: Curtis Suttle

INSTITUTION: University of Texas at Austin, Marine Science Institute

GRANT TITLE: Development and use of Fluorescent Probes for Detection, Enumeration and Identification of Naturally-occurring Marine Viruses

REPORTING PERIOD: 1 October 1989 to 31 May 1991 (20 months)

AWARD PERIOD: 1 October 1989 to 30 September 1991

OBJECTIVES: The major research objectives are as follows:

- To isolate representative marine virus systems
- To develop taxon-specific probes for marine viruses
- To determine spatial and temporal distributions of specific viral taxa
- To establish a library of natural marine virus communities and a culture collection of marine viruses and their hosts
- To characterize the viruses that we isolate

ACCOMPLISHMENTS AND SIGNIFICANCE: Substantial progress has been made towards realizing the stated objectives. We have isolated viruses that infect a number of phytoplankton including a prasinophyte (Micromonas pusilla), pennate (Navicula sp.) and centric (of uncertain taxonomy) diatoms and a cyanobacterium (Synechococcus sp). These represent some of the major taxa of primary producers in the sea. As well, we have isolated a number of bacteriophage, including several which infect bioluminescent bacteria. These virus-host systems should provide excellent models for addressing basic questions such as whether viruses can control the distribution and abundance of planktonic organisms.

To determine if viruses might affect primary productivity, material in the 0.002 to 0.2  $\mu\text{m}$  size range was concentrated from seawater using ultrafiltration and added to natural phytoplankton communities. Within minutes photosynthetic rates were reduced by up to 80 % and over the longer term in vivo chlorophyll fluorescence decreased. Although the reduction in photosynthesis cannot be definitively attributed to viruses, the causative agent was filterable through a 0.2  $\mu\text{m}$  filter and was sensitive to autoclaving, consistent with a viral-mediated process.

We chose a couple of these systems as models to examine spatial and temporal distributions of marine viruses. The great abundance and diversity of viruses in the sea, combined with generally low titers of specific types of viruses, suggests that there may be  $10^5$ - $10^6$  different kinds of viruses  $\text{ml}^{-1}$  of seawater! For these reasons and because of the variability in specificity, fluorescently-labelled polyclonal antibodies are not adequate for enumerating viruses in seawater. Instead, we have used plaque or most probable number (MPN) assays. As well, we hope to identify gene sequences that will allow us to easily distinguish between different types of viruses that infect the same host. We have found that specific viruses are widely distributed and titers are highly variable. For example, we isolated viruses infecting M. pusilla from the Pacific and Atlantic oceans, and the Gulf of Mexico. Titers of the virus varied from thousands per ml to one per 30 ml. Although the viruses appeared identical they were genetically and phenotypically distinct based on DNA

restriction fragment analysis and molecular weights of the major proteins. This was true of viruses isolated from the same water sample! These results indicate the high genetic diversity and dynamic nature of virus populations in the sea.

Ultrafiltration has also been used to create a library of natural virus communities. Viruses are concentrated from seawater obtained from different locations and times, and then preserved at -80° and 4° C. The library is a source for new virus isolates or can be screened to determine if a specific virus is present at a given location and time. It is anticipated that the library will be a very valuable asset as probes become available for more types of viruses. Currently, we have over 40 virus communities preserved.

Considerable effort has been spent characterizing the viruses. The cyanophage infecting Synechococcus is typical of the Styloviridae which infects freshwater cyanobacteria. Yet, the virus is very host specific and did not infect any of eleven Synechococcus strains tested. The high host specificity of cyanophages is likely why Synechococcus can exist in high concentration in the sea. The viruses infecting M. pusilla are large untailed polyhedrons. It is not clear if they belong to any known class of viruses. The diatom viruses are different. Although the infective agent has been partially purified, electron microscopy has not revealed any obvious viral-like particles. Some of the bacteriophages have also proved to be unusual. Pulse-field gel electrophoresis of intact phages suggests that one clone may be highly polymorphic, with perhaps as many as ten different size particles. Results such as these indicate that the sea will provide a panacea of novel viruses.

WORK PLAN (next 6 months): We will continue with characterization of the viruses we have isolated, add to our library of natural virus communities and search for conserved regions of viral DNA to which probes might be made.

#### PUBLICATIONS AND REPORTS:

1. ONR-supported work was published in two refereed journals.  
Suttle, C.A., A.M. Chan, and M.T. Cottrell. 1990. Infection of viruses by phytoplankton and reduction of primary productivity. *Nature* 347:467-469.  
Suttle, C.A., A.M. Chan and M.T. Cottrell. 1991. Use of ultrafiltration to isolate viruses from seawater which are pathogens to marine phytoplankton. *Applied and Environmental Microbiology*, 57:721-726.
2. Two abstracts have been published and one submitted  
Suttle, C.A., A.M. Chan and M.T. Cottrell. 1990. Viruses infect marine phytoplankton and reduce primary productivity by up to 80%. *EOS* 71:162.  
Suttle, C.A. 1991. Response of phytoplankton communities to addition of material in the 30,000 to 0.2  $\mu\text{m}$  size fraction concentrated by ultrafiltration *Abs. Am. Soc. Limnol. Oceanogr.*  
Suttle, C.A. 1991. (invited). Abundance, diversity and nature of virus communities in the sea. *Intern. Phycol. Congr.*

### **METAL ION BIOSENSORS**

ARI (JOINT WITH CHEMISTRY, OCEANIC BIOLOGY, AND ONT)

SCIENTIFIC OFFICER: DR. HAROLD J. BRIGHT

PROGRAM OBJECTIVE: TO DESIGN REUSABLE BIOSENSORS FOR REAL-TIME QUANTIFICATION OF METALS (GROUP IIB AND THE DIVALENT TRANSITION METAL IONS) PRESENT AT LOW LEVELS (OFTEN  $\leq 10^{-9}\text{M}$ ) IN THE MARINE ENVIRONMENT.

NAVY OBJECTIVE: TO DEVELOP A SCIENTIFIC BASIS FOR IMPROVED SENSORS CAPABLE OF MEASURING AND MAPPING NUTRIENT LEVELS IN THE SEA, ASSESSING ECOLOGICAL FACTORS AND THEIR VARIATION, MEASURING TOXIC MATERIALS RELEASED IN THE SEA, AND FOR MONITORING WATER QUALITY IN AND AROUND NAVAL BASES OF OPERATION.



## FINAL PROGRESS REPORT

GRANT #: N00014-88-K-0469

PRINCIPAL INVESTIGATOR: Frances H. Arnold

INSTITUTION: California Institute of Technology

GRANT TITLE: Design and Construction of Synthetic Metal-Binding Proteins

PERIOD OF PERFORMANCE: 1 July 1988 - 30 June 1991

OBJECTIVE: To investigate selective metal recognition by proteins using site-directed mutagenesis to construct simple metal-binding motifs.

### ACCOMPLISHMENTS:

Metal-binding sites consisting of two histidines positioned His  $X_3$ -His in an  $\alpha$ -helix have been engineered into *S. cerevisiae* cytochrome c (Caltech) and bovine somatotropin (Monsanto Co.). When placed in this configuration, nitrogens of the imidazole rings are well positioned to chelate metal ions. Using a new technique developed in the laboratory, metal-affinity partitioning in aqueous two-phase systems [1], we have measured stability constants for the interaction between the engineered proteins and Cu(II);IDA. Association constants of  $10^4$  to  $10^6$  M<sup>-1</sup> have been obtained for the formation of the protein-Cu(II)-polymer complexes with the various His- $X_3$ -His proteins, as reported in Ref. 3.

Simple metal-chelating motifs consisting of two histidines in other elements of secondary structure can also be found. Modeling calculations for first-row transition metals such as Cu(II) show that selected short amino acid sequences coupled with appropriate elements of secondary structure can fulfill the requirements for metal chelation (Table 1). Di-histadine configurations His- $X_1$ -His in an  $\alpha$  helix, His- $X_2$ -His in a reverse  $\beta$  turn, and His- $X$ -His in a  $\beta$  strand for chelating sites. The geometric requirements for chelation are highly specific: while two histadines separated by three amino acids in an undistorted  $\alpha$  helix can chelate a metal ion, histadines separated by two or four residues do not. The number of practical metal-chelating configurations in common secondary structure elements is very limited. Although additional chelating configurations can be found in other types of structured regions of proteins, the simple motifs in Table 1 are generally applicable for engineering metal-binding sites into proteins.

Two histidines placed across a  $\beta$ -sheet can also chelate metal ions. We are currently testing the His- $X_2$ -His site in a  $\beta$ -sheet and across the  $\beta$ -sheet in cytochrome c. The following mutants are being constructed: Gly 37-His, Asn 56-His (across sheet from His 39), and Asn 58-His (also across sheet from His 39). Characterization of these variants will allow us to determine the metal-chelation contribution from di-histadine sites in  $\beta$ -sheet structures.

**Table 1.** Possible metal-chelation sites in secondary structure elements:  $\alpha$  helix,  $\beta$  strand, and reverse  $\beta$  turn (type I' and II'): (+) Chelation cannot occur. Bonding parameters are  $M-N = 2.01 \pm 0.02 \text{ \AA}$ ,  $N-M-N = 75^\circ$  to  $115^\circ$ .

Sequence	$\alpha$ Helix	$\beta$ Strand	Reverse $\beta$ turn
HH	-	-	-
HXH	-	+	-
HX <sub>2</sub> H	-	-	+
HX <sub>3</sub> H	+	-	-
HX <sub>4</sub> H	-	-	-

*Metal binding as a sensitive probe of protein structure.*

For a good chelating di-histadine site, binding constants are two orders of magnitude greater than those for the same copper complex binding to a similar protein with two exposed, non-chelating His residues. Observed values of  $\Delta\Delta G^\circ_{\text{chelate}}$  range from 0.9 to 3.5 kcal mol<sup>-1</sup> for different His-X<sub>n</sub>-His sites and reflect both the high sensitivity of metal binding to local structure and the differences in the structures and stabilities of helices in proteins. Without a stable structural framework to provide a rigid base for formation of the chelate complex, chelation does not occur. Consequently, the metal binding strength of a chelating site is also highly sensitive to environmental conditions that alter its structure or stability. For example, the chelating His 4-His 8 site in the NH<sub>2</sub>-terminal helix of cytochrome c loses its high affinity for soluble Cu(II)IDA-PEG above pH 8.0, even though the protein remains folded up to pH 12 [4]. Furthermore, this His-X<sub>3</sub>-His site in the NH<sub>2</sub>-terminal cytochrome c helix exhibits a relatively low affinity for Cu(II)IDA immobilized on a solid support. The loss of the protein scaffolding contribution at high pH or upon adsorption to a surface, clearly mirrored in the ability of this site to chelate Cu(II), must arise from small changes in the structural integrity of the NH<sub>2</sub>-terminal helix. This high sensitivity to environmental conditions is potentially useful in designing agents with readily reversible metal-binding characteristics.

*Incorporation of simple metal-binding sites as general means of protein stabilization.*

In addition to their use in purification and in probing local conformation, engineered metal-chelating sites are effective in stabilizing folded proteins. Metal ions can shift the thermodynamic folding-unfolding equilibrium for a peptide or protein by binding preferentially to the folded form. By virtue of its ability to rigidly position the histadine ligands to promote metal chelation, the protein contributes a binding free energy (the "scaffolding" contribution),  $\Delta\Delta G^\circ_{\text{chelate}}$ , which varies from 0.9 to 3.5 kcal/mol for a single His-X<sub>n</sub>-His site binding Cu(II) [3]. Using simple thermodynamic arguments, it can be shown that a metal ion that binds preferentially to the folded protein will stabilize the protein by an amount equivalent to  $\Delta\Delta G^\circ_{\text{chelate}}$  [5,6]. Thus metal ions can stabilize existing or incipient structures: metal chelation by a His-X<sub>n</sub>-His can lock in a marginally stable helix or even add to the global stability of the folded protein. For proteins

that obey a simple two-state folding mechanism,  $\Delta\Delta G^{\circ}_{\text{cholate}}$  is equal to  $\Delta\Delta G^{\circ}_{\text{unf}}$ , the difference in the free energy of unfolding in the presence and absence of the metal complex. This quantity is the degree to which metal chelation stabilizes the folded protein. In fact Cu(II)IDA stabilizes the His 4--His 8 cytochrome c variant to denaturation by guanidinium hydrochloride by the same free energy as its (independently measured) chelating contribution to metal binding [5,6]. The addition of Cu(II)IDA to a variant containing a single His-X<sub>3</sub>-His site can realize up to 4kcal mol<sup>-1</sup> of stabilization, an appreciable quantity in view of the fact that many folded proteins are more stable than their unfolded forms by only 5 to 15 kcal mol<sup>-1</sup>. Addition of free copper or other metal complexes that have higher binding energies, or the incorporation of multiple chelating sites into the protein could provide even greater stabilization. Provided that the substitution of surface residues by histadine does not drastically reduce stability, engineered chelating sites provide a generally applicable mechanism for enhancing protein stability at elevated temperatures or in the presence of denaturants such as organic solvents.

#### SIGNIFICANCE:

The goal of this research has been to identify elements of metal-binding functions that can be used to create synthetic metal-binding proteins. We have demonstrated important design elements for synthetic metal-binding proteins--most importantly, that the binding site must be engineered into rigid sections of the protein to minimize entropic costs of chelation [3]. Simple motifs consisting of only two histadines positioned in chelating configurations in commonly elements of secondary structure can provide high affinity for metal ions. We have been able to demonstrate several applications for these simple sites, including protein purification [4], as sensitive probes of protein structure [4,5] and in stabilizing proteins [5; Kellis and Arnold, in preparation].

#### PUBLICATIONS AND REPORTS (last 12 months):

1. "A Mathematical Model for Metal Affinity Partitioning," S.-S. Suh and F.H. Arnold, *Biotechnol. & Bioeng.*, **35**, 682-690 (1990).
2. "Metal-Affinity Separations: A New Dimension in Protein Bioprocessing," F.H. Arnold, *Bio/Technology*, **9**, 151-156 (1991).
3. "Characterization of His-X<sub>3</sub>-His Sites in  $\alpha$ -Helices of Synthetic Metal-Binding Bovine Somatotropin," S.S. Suh, B.L. Haymore and F.H. Arnold, *Protein Engineering*, **4**, 301-305 (1991).
4. "Cu(II)-Binding Properties of a Synthetic Metal-Binding Cytochrome c: His-X<sub>3</sub>-His in an  $\alpha$ -Helix," R. Todd, M. Van Dam, D. Casimiro, B.L. Haymore, and Arnold, F.H. *Proteins: Struct. Funct. Genet.*, **10**, 156-161 (1991).
5. "Protein-Metal Interactions: Purification to Protein Folding," F.H. Arnold and B.L. Haymore, *Science*, **252**: 1796-1797 (1991).

# ANNUAL PROGRESS REPORT

GRANT#: N00014-90-J-1700

PRINCIPAL INVESTIGATOR: Jeremy M. Berg

INSTITUTION: The Johns Hopkins University

GRANT TITLE: Ligand Field Stabilization Energy Control of Metal Ion Binding

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 15 March 1990 - 14 March 1993

OBJECTIVE: To investigate the role of changes in ligand field stabilization energy in determining the relative affinities of metal ions to metal-binding peptides and proteins.

ACCOMPLISHMENTS (last 12 months): Considerable progress has been made in three areas over the past year: (1) Refinement of methods for determining relative affinities of metal ions to peptides; (2) Extension of metal binding studies to other metal ions; and (3) Preparation and characterization of new peptides for binding studies. These will be discussed in turn.

(1) We had previously determined the relative affinities of a given metal ion to a series of peptides by determining the dissociation constant for each metal-peptide complex in a separate experiment and comparing the results. This method has the advantage of simplicity but suffers the significant drawback the uncertainties in the two determinations are uncorrelated so that the error in the ratio of dissociation constants is relatively large. We have developed an alternative method based on competition experiments. A mixture of the two peptides is titrated with metal ion and absorption spectra are recorded. These spectra are the deconvoluted based on the known spectra of the two components and the relative amounts of each complex is determined. Much more precise determinations of the relative affinities of Co(II) to the peptides CP-1, CP-1(CCHC), and CP-1(CCCC) have been achieved using these methods. CP-1 has the sequence ProTyrLys**Cys**ProGlu**Cys**GlyLysSerPheSerLysGlnSerAspLeuValLys**His**GlnArg-Thr**His**ThrGly with the ligands shown in bold and CP-1(CCHC) and CP-1(CCCC) have one or both of the His residues replaced with Cys. The results of these studies have revealed that changes in ligand field stabilization energy are an important factor in determining the relative affinities of these peptides for Zn(II) over Co(II) although other terms must also contribute.

(2) We have extended our studies with these peptides to include the additional metal ions Fe(II), Ni(II), Mn(II), and Cd(II). Fe(II) and Ni(II) form well-defined complexes with the three peptides. Binding studies have been completed with CP-1 and CP-1(CCHC). These peptide-metal complexes all have dissociation constants in the range of  $10^{-6}$  M, approximately four orders of magnitude higher than the corresponding Zn(II) complexes. The absorption spectra are consistent with the expected cysteinate, histidine coordination with distorted tetrahedral geometries. The nature of the CP-1-Ni(II) complex has been examined further with use of  $^1\text{H}$  nuclear magnetic resonance spectroscopy. The spectrum clearly indicates that the complex is paramagnetic as expected

for a tetrahedral but not for a square planar complex. Mn(II) was found not to be bound by CP-1 or its derivatives with high affinity. In contrast, Cd(II) was bound by all three peptides with the affinity increasing greatly as the number of thiolate ligands increased.

(3) A derivative of CP-1 in which that final four residues from the carboxyl terminus have been deleted has been synthesized and characterized. This peptide binds metal ions such as Co(II) and Zn(II). The Co(II) complex is tetrahedral with a water acting as the fourth ligand. Metal ion titrations of the peptide are complicated by the fact that it forms 2:1 peptide to metal complex at high peptide to metal ratios. Nonetheless, methods have been developed for determining the dissociation constants for both the 1:1 and 2:1 complexes based on spectra deconvolution methods. The 1:1 complex will bind a number of exogenously added ligands such as beta-hydroxyethanethiolate, N-methylimidazole, and chloride. The absorption spectra of these complexes are consistent with the expected structures. A derivative in which the remaining His is replaced by Cys has also been synthesized.

SIGNIFICANCE: The improvements of our relative dissociation constant determination methods will be of great utility in the remainder of our studies. The results with Co(II) and Zn(II) indicate that ligand field stabilization energy effects are a major, but not the only, term that determines relative ion affinity. Our observations with other metals are preliminary but quite informative. Simple ligand field stabilization energy arguments suggest that Ni(II) should be bound very weakly in a tetrahedral site. The fact that Ni(II) binds reasonably tightly to our peptides indicates that this effect is greatly reduced either by the inherent lack of symmetry in the site or metal-induced distortion. In contrast, based on ligand field stabilization energy effects alone, Mn(II) would be expected to be bound tightly. The fact that only weak binding is observed reveals the importance of other terms such as hard/soft acid/base effects. Finally, the availability of the peptide with an open coordination site should allow us to examine ligand binding as a function of metal ion and of the nature of the remaining ligands.

WORK PLAN (next 12 months): Our highest priority for the next year is to complete our metal binding constant determinations for the peptides and metals discussed above. These data should allow us to evaluate how important the ligand field stabilization energy effects are in determining relative ion affinities for this series of metal ions. In addition, we plan to prepare peptides in which the final histidine in CP-1 is replaced by other potential ligands such as glutamic acid, aspartic acid, and methionine to determine if these residues will, indeed act as ligands. If they do bind appropriate metals, measurements of metal-peptide complex dissociation constants will be performed.

INVENTIONS: None

PUBLICATIONS:

1. Denis L. Merkle, Michael H. Schmidt, and Jeremy M. Berg, "Design of a Ligand-Binding Metallopeptide", Journal of the American Chemical Society, 1991, in press.

# ANNUAL PROGRESS REPORT

Grant #: N00014-90-J-1711

R&T Code: 441s005

PRINCIPAL INVESTIGATOR: Thomas C. Bruice

INSTITUTION: University of California at Santa Barbara

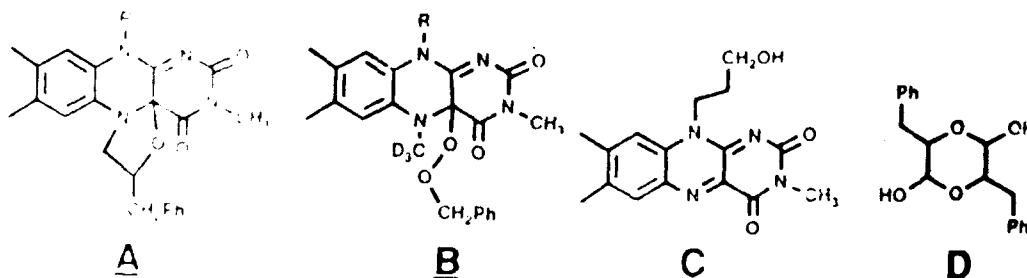
GRANT TITLE: The Synthesis of Chemiluminescent Flavoabzymes and Their Use in Metal Detection

REPORTING PERIOD: 15 March 1990 to 24 May 1991

AWARD PERIOD: 15 March 1990 to 14 March 1992

OBJECTIVE: There are four objectives which must be reached in sequential order. The 1st objective is to synthesize a molecule (hapten) which resembles, in space requirements and structure, a 4a-alkyl hydroperoxide adduct of a 4a,5-dihydroflavin. The 2nd objective is to prepare a protein conjugate with this hapten and with this antigen, proceed to the preparation of monoclonal antibodies (MAbs). The third objective is to identify one or more MAbs that catalyze a chemiluminescent reaction of alkyl hydroperoxide and flavin. This would be a flavoabzyme which catalyzes a reaction known to be catalyzed by bacterial luciferase. The 4th objective will then be to modify the hapten such that the MAbs become specific complexers of metal ions and to determine the change in quantum efficiency in the presence of metal ions.

ACCOMPLISHMENTS: The first phase of the study as it was initially planned involved approaches to the synthesis of a molecule (structure A) which has the space requirements of a 4a-benzyl hydroperoxide adduct of a 4a,5-dihydroflavin (structure B) and also has a "tail" (R) that can be attached to the carrier keyhole limpet hemocyanin protein to form the antigen.



The first synthetic sequence to be attempted was based on the knowledge that silylation with Ph<sub>2</sub>(*tert*-Bu)SiCl occurs only with primary alcohols and a report<sup>1</sup> in the literature that methylation of all hydroxyl groups of riboflavin was achieved by treatment of the latter with (AgO + MeI)/DMF. The silylation reaction was successful (85%); however, we were not able to carry out the methylation reaction. We then turned to the use of N(3)-methyl-N(10)-hydroxyethyl lumiflavin (C) as a starting material. We showed<sup>2</sup> some time ago that a clean procedure for the N(5)-alkylation of lumiflavin (Fl<sub>ox</sub>) is through the formation of an imine with 1,5-dihydroalumiflavin (FlH<sub>2</sub>) followed by reduction. With the idea to adapt this general procedure, the aldehyde [(S)-PhCH<sub>2</sub>CH(OH)CHO] was prepared (20%) but found to exist as the unreactive diaxane (D). The formation of D was prevented by benzylation of the hydroxyl group of (S)-PhCH<sub>2</sub>CH(OH)CHO and initial attempts using the

benzylated material will be further pursued. The reductive debenzylation may not proceed with facility, in which case the para-MeOC<sub>6</sub>H<sub>4</sub>CH<sub>2</sub>- blocking group will be employed. Succinylation of the hydroxyethyl function at N(10) would provide the target molecule. An alternate route follows from our synthesis of (S)-PhCH<sub>2</sub>CH(OH)CH<sub>2</sub>I and the use of this reagent in the alkylation of the appropriate 1,5-dihydroflavin species. Additional work is required with this path.

References:

1. D. B. McCormick, *J. Heterocyclic Chem.*, 1974, 11, 969.
2. S. Ball; T. C. Bruice, *J. Am. Chem. Soc.*, 1981, 103, 5494

SIGNIFICANCE: No significant results have been obtained at this date.

WORK PLAN: We will continue in the directions outlined in the original grant request as indicated in the ACCOMPLISHMENTS section. The target molecule is sterically attainable.

PUBLICATIONS AND REPORTS (last 12 months): None.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-3037

R&T CODE: 400x066

PRINCIPAL INVESTIGATOR: David W. Christianson

INSTITUTION: University of Pennsylvania

GRANT TITLE: Structural Studies of Metalloproteins:  
Recognition and Specificity of Ligand Binding and Metal Ion  
Association

REPORTING PERIOD: 1 July 1990 - 30 June 1991

AWARD PERIOD: 1 July 1989 - 30 June 1992

OBJECTIVE: To understand the general structure principles (short-range and long-range) that lead to recognition and binding of specific metal ions by proteins (i.e., metal ion biosensors), and to understand how metalloenzymes recognize and bind certain ligands.

ACCOMPLISHMENTS (last 12 months): In the past year, we have completed the first phase of an architectural study of zinc coordination stereochemistry as the metal ion is liganded by various functional groups (Christianson, 1991), and as the direct metal ligands are nested within greater hydrogen bond networks (Alexander and Christianson, 1990; Ippolito et al., 1991; Christianson, 1991). The assembled structural information guides protein engineering experiments on the zinc metalloenzyme carbonic anhydrase II. We have completed studies in which (1) the active site hydrophobic pocket has been rationally engineered (Alexander et al., 1991; Nair et al., 1991), (2) catalytic residues have been probed (Krebs et al. 1991,) and (3) the pH-dependent conformation of His-64, an active site residue and potential metal ligand, has been explored (Nair and Christianson, 1991). Other in-progress work includes the study of enzyme-peptidylsulfonamide and enzyme-gas complexes.

SIGNIFICANCE: The X-ray crystallographic experiments yield valuable information regarding the compensatory accommodation of point mutations in the protein scaffolding. For example, in response to mutations at a partially-buried residue, we observe compensatory expansions/contractions of the protein scaffolding that propagate 10-15 Å from the point of mutation. For a mutation at a solvent-exposed residue, we see in one example a compensatory conformational change at another residue 6 Å away mediated by slight changes in solvent structure. These effects must be fully understood in order to improve the stereochemical rationale guiding the construction of mutant plants.



WORK PLAN (next 12 months): Mutant carbonic anhydrases II, where amino acid substitutions have been made to zinc ligands, are currently set up for crystallization in our laboratory. For example, the His-94 Cys mutant exhibits optimal cysteine-zinc coordination stereochemistry based on modeling experiments (Christianson, 1991), and the solution of its crystal structure will reveal how closely our structural model matches reality (His-119 Cys. will also be studied). Importantly, we also target Asn-244 Asp and Gln-92 Glu mutants for study; these residues hydrogen bond with histadine zinc ligands in the wild-type enzyme, and these hydrogen bond networks contribute to protein-metal affinity as well as transmit metal binding "signals" through the protein scaffolding. It is our tenet that the entire protein, and not simply the inner-sphere metal ligands, serves as the metal ligand, we note that our approach is unique, where we manipulate an existing zinc binding site according to precise stereochemical rules.

PUBLICATIONS AND REPORTS (last 12 months):

Christianson, D.W. and Alexander, R.S. (1990) "Another Catalytic Triad?" Nature 346, 225.

Ippolito, J.A., Alexander, R.S., and Christianson, D.W. (1991) "Hydrogen Bond Stereochemistry in Protein Structure and Function". J. Mol. Biol. 215, 457-471.

Kanyo, Z.F. and Christianson, D.W. (1991) "Recognition of Biological Pyramids: Phosphate and Sulfate". J. Biol. Chem. 226, 4264-4268.

Christianson, D.W. "The Structural Biology of Zinc". Adv. Prot. Chem. (in press)

# ANNUAL PROGRESS REPORT

GRANT #: N0001490-J-1694

R&T CODE: 441s002

PRINCIPAL INVESTIGATORS: Andrew Hamilton and Stephen Weber

INSTITUTION: University of Pittsburgh

GRANT TITLE: Divalent Transition Metal Sensors

REPORTING PERIOD: 1 March 1990 - 30 May 1991

AWARDING PERIOD: 1 March 1990 - 28 February 1993

OBJECTIVE: To develop metal chelators that show a change in fluorescence upon binding to metal IIB dications and other transition metal dications; to develop photo- or electroswitchable metal binding molecules. Incorporation of switch into peptides and related models; intra- and intermolecular control of peptide conformation.

ACCOMPLISHMENTS: We have discovered a molecule of the spirobenzoxazone class that forms complexes with Zn(II), Cd(II), and Hg(II) upon irradiation with low energy UV light (Figure 1). The complexes formed are highly absorbing and fluorescent. The reactions have been carried out in acetonitrile, and other divalent transition metal ions show some reactivity. Exposure to light in the absence of divalent metal ions leads to blue colored solution that is not luminescent (to the eye) and that rapidly fades. The photochemical reaction products with metals are stable for hours, and all the reactions are reversible. This and similar compounds have been made available to us by PPG. Chemicals research.

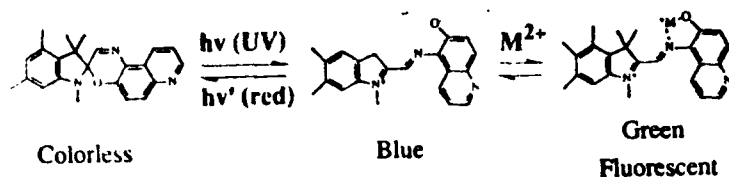


Figure 1

We have done the first electrochemical investigations of the photochromic spirobenzopyrans (abbreviated BIPS). The 6-hydroxy-BIPS is photochromic, but it is also electrochromic; the product of the oxidation is probably the quinone (Figure 2). Attempts to electrochemically trap the photoisomerized product are underway.

We have also made excellent progress in the area of protein secondary structure stabilization. We have shown that synthetic molecules with appropriately positioned binding sites can alter peptide conformations in a well-defined way (intermolecular stabilization). We have also developed a photochemical method involving cyclobutane formation between cinnamoyl esters of two (i+4) serines, for covalently cross linking  $\alpha$ -helices (intramolecular stabilization). This approach has been shown to work in small pentapeptides.

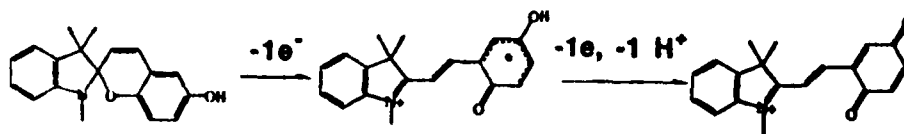


Figure 2

SIGNIFICANCE: To our knowledge, we have discovered the first photoreactive, metal sensitive luminescent compound. There are clear applications in metal ion sensing. The first comprehensive investigation of the electrochemistry of the spirobenzopyrans will lead to complete control over the course of reactions involving these photochromics. Consequently, when these photochromics are incorporated into peptide that bind to metals, their reactivity and stability will be under our control. Applications in sensing and other technologies are anticipated.

Our intra- and intermolecular approaches to protein stabilization will provide additional control over the switched metal ion binding process. Beyond this, protein stabilization and recognition are areas of enormous fundamental importance. Our work is among the first to tackle these problems in a well-defined, molecular approach, and should yield new insights into protein folding and protein-small molecule interactions.

WORK PLAN (next 12 months): We will investigate the properties of the metal binding benzoxazones in two phase systems, one being water and the other containing the water insoluble active compound. We understand multiple phase equilibria, (see publication list) and will apply that rationale to the choice of the second phase. This second phase may be cellulose acetate because it is stable to seawater. Other analogous photochromics will be surveyed for their reactivity towards the target metals.

We will complete a full study of the electrochemistry of the spirobenzopyran class, and in particular of the 6-hydroxy compound which has a stable and reversible product form, the quinone. We will functionalize this molecule to give it biomimetic metal binding properties, and reinvestigate the photochemistry and electrochemistry in the presence of the target metal ions.

We will extend our peptide photochemical cross linking method to longer peptides. A series of 15-20 mers including two serine-cinnamoyl derivatives will be prepared and the conformation of the irradiated product will be determined by spectroscopic methods. We will continue our studies of intermolecular control of peptide conformation by focusing on secondary structural units such as  $\alpha$ -helices. We will attach the photoswitchable units to peptides to determine their influence on metal ion binding.

PUBLICATIONS AND REPORTS (last 12 months):

1. Sensor-related papers; work completed under ONR support. Kuhn, L.S. and Weber, S.G. (1991), Novel solvent systems for the preparation of phase-inversion cellulose acetate size exclusion membranes: Voltammetric investigations, accepted: Electroanalysis. Hinze, W.L. and Weber, S.G. (1991), Why the relationship between the logarithm of  $k'$  and homolog number in micellar chromatography is not linear, accepted: Anal. Chem.
2. Abstract of spiropyran work, Pittsburgh Conference 1991. Preigh, M.J. and Weber, S.G. (1991) Electrochemical control of spiropyran: Mechanism and potential application to metal ion detection.
3. Peptides: Vicent, C., Hirst, S.C., Garcia-Tellado, F. and Hamilton, A.D. (1991), Conformational selectivity in molecular recognition: The influence of artificial receptors on the cis-trans isomerization of acyl-prolines, J. Am. Chem. Soc., in press.

## ANNUAL PROGRESS REPORT

GRANT#: N0001490WX24248

R&T CODE: 441g001

PRINCIPAL INVESTIGATOR: Linda I. Hannick, Ph.D.

INSTITUTE: Laboratory for the Structure of Matter, Naval Research Lab

GRANT TITLE: Photoproteins as Metal Ion Biosensors

PERIOD OF PERFORMANCE: 1 June 1990 - 31 May 1991

OBJECTIVE: To investigate the structures and mechanisms of the proteins responsible for light emission in the bioluminescent system of the jellyfish *Aequorea aequorea*.

### ACCOMPLISHMENTS:

#### **I. Aequorin**

**Purification and Crystallization of Aequorin**: We have extracted recombinant apo-aequorin from *E. coli* cell paste. The material was renatured, charged with synthetic chromophore and purified. From this material, diffraction quality crystals were prepared by the vapor diffusion method. These crystals are amber plates, 1.4mm x 1.2mm x 0.76mm in size, forming in interdigitated layers that separate upon probing into single crystals (Figure 1). When immersed in a solution of  $\text{CaCl}_2$ , the crystals luminesce strongly for over twenty-four hours, and generate small bubbles ( $\text{CO}_2$ ?) on the surface, and gradually disintegrate. This confirms that they are composed of active photoprotein.

**X-ray diffraction results**: The crystals diffract to beyond 2 Å resolution, are very stable in the x-ray beam and are not temperature sensitive. They exhibit the symmetry of space group  $P2_12_12_1$ , with  $a=89.1$ ,  $b=88.4$ ,  $c=52.7$  Å,  $\alpha=\beta=\gamma=90^\circ$  and two molecules per asymmetric unit (a.u.). A data set was collected on these crystals, with 96,596 observations resulting in 10,657 reflections.

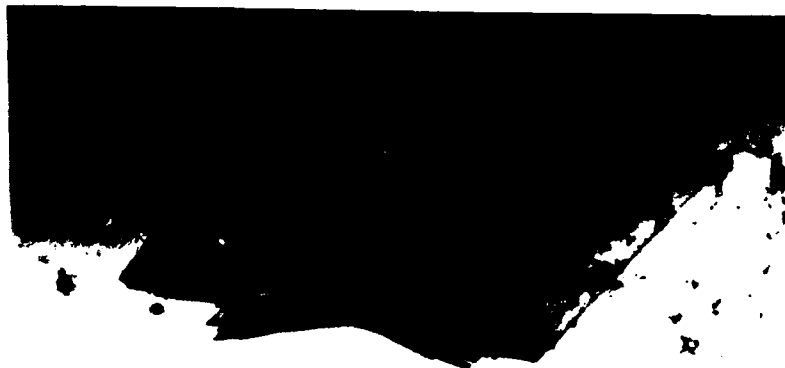
**Structure Solution**: Initial analysis of the data, using the self-rotation function, resulted in the determination of a non-crystallographic dyad axis. This is evidence for a two molecules in the a.u., which is in agreement with the calculation of solvent density of 42%. An attempt is currently being made to phase the data using the coordinates of a model protein which is closely related to aequorin, Sarcoplasmic Calcium-binding Protein. In addition, the search for heavy-atom derivatives has begun.

#### **II. Green Fluorescent Protein (GFP)**

**Material preparation and purification**: We collected and processed 65,000 jellyfish for a crude protein yield of approximately 800 g. New purification procedures are being developed to separate the five

isoproteins more completely.

**Crystallization and diffraction:** Crystals were grown using purified L isoprotein. Both hexagonal and needle-like forms of crystals were obtained. The previously prepared monoclinic crystals, which are optimum for diffraction analysis, did not grow from this preparation. Data collected on the hexagonal form indicates that these crystals diffract to 3.5 Å, but data are of poor quality due to a large (412 Å) lattice constant.



**Figure 1.** Photograph (@7x magnification) of crystals from sitting drops of aequorin. Size: 1.4mm x 1.2mm x 0.06mm

**SIGNIFICANCE:** The structural analyses of these proteins will provide insight into the mechanism by which light is emitted following metal binding. Future modifications to the structure could result in novel metal detecting proteins.

**WORK PLAN:** The objectives during the next year are to continue x-ray crystallographic analysis of recombinant aequorin and of native GFP. The search for heavy-atom derivatives for both proteins will continue. Additional *Aequorea* jellyfish will be collected in September to obtain native protein to continue the GFP work.

**INVENTIONS:** no patentable inventions resulted from this segment of the project.

#### PUBLICATIONS AND REPORTS

*PlotHKL: A Program to Depict Planes of Diffraction Data Collected using the Siemens Area Detector or Diffractometer*, N. Pattabiraman, L. I. Hannick\*, and Keith B. Ward, presented at the 3rd Annual Siemens Area Detector Users Meeting, La Jolla, CA, May 5-7, 1991

*A PC-based Spreadsheet for Tracking Results of Crystallization Experiments*, L.I. Hannick, M. A. Perozzo, and K. B. Ward, Fourth International Conference on Crystal Growth of Biological Macromolecules, Freiburg, Germany, August 18-24, 1991.

*Preparation and Initial Characterization of Crystals of the Photoprotein Aequorin from Aequorea aequorea*, Hannick, L.I., Prasher, D.C., Schultz, L.W., Deschamps, J.R. and Ward, K.B., manuscript in preparation.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1713

R&T CODE: 441s004

PRINCIPAL INVESTIGATOR: Barry Honig

INSTITUTION: Columbia University

GRANT TITLE: Analysis and Design of Metal Binding Sites

REPORTING PERIOD: 15 March 1990 - 14 May 1991

AWARD PERIOD: 15 March 1990 - 14 March 1993

OBJECTIVE: The overall objective of this proposal is to develop computational methods for the design of metal binding sites in proteins. In particular, we would like to engineer a zinc binding site into a calcium binding protein.

ACCOMPLISHMENTS (last 12 months): The standard strategy being used to predict the structure of small regions in proteins is to generate a large number of trial structures and then to select a subset of lowest energy. In the past year we began working on the loop prediction problem for calcium binding sites. Using an 8 residue loop as an example, we generated about 500 conformations and attempted to evaluate the conformational energy of each. In general, we were not able to identify energetic criteria that successfully picked out the observed crystal conformation. This suggests that the energy functions used in model building studies are fundamentally flawed. The underlying problem is that solvent effects are not properly accounted for. We have begun to address the problem by developing improved treatments of electrostatic and hydrophobic free energies. In particular we have found, by analyzing solubility data, that the hydrophobic effect is about twice as large as previously suspected.

SIGNIFICANCE: Our discovery concerning the magnitude of the hydrophobic effect is of great general significance and, in particular, should have far-reaching implications on model building studies of proteins. Our loop generation work is an important first step in developing general methods to predict protein conformation. This will have an impact on many of the current ONR grantees who are attempting to design metal binding sites.

WORK PLAN (next 12 months): The objective for the next year is to integrate accurate energy evaluation methods into model building studies. This will include continuum electrostatic calculations and surface area relationships to account for hydrophobicity. The work will involve a rather major effort since a large number of possible conformations

must be considered and thus the calculations become quite time-consuming. We will attempt to account for the conformation of specific calcium binding sites and begin to consider how to design zinc ligands into these sites.

PUBLICATIONS AND REPORTS (last 12 months):

Sharp, K., Nicholls, A., Friedman, R., and Honig, B.

Extracting hydrophobic free energies from experimental data: Relationship to protein folding and theoretical models. (Submitted to Biochemistry).

Nicholls, A., Sharp, K., and Honig, B. Protein folding and association: Insights from the interfacial and thermodynamic properties of hydrocarbons.

# ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-3038

R&T CODE: 400x067yip02

PRINCIPAL INVESTIGATOR: Daniel E. Kahne

INSTITUTION: Princeton University

GRANT TITLE: Understanding how Small Molecules Cleave DNA Site-Specifically: The Role of the Carbohydrate Tail of Calicheamicin.

REPORTING PERIOD: 1 July 1990 - 30 June 1991

AWARD PERIOD: 1 July 1989 - 30 September 1991

OBJECTIVE: To investigate the role that the carbohydrate portion of calicheamicin plays in DNA recognition with the ultimate aim of utilizing what we learn to design other sequence-specific DNA binders.

ACCOMPLISHMENTS: We have completed the total synthesis of the calicheamicin oligosaccharide. All glycosidic linkages were constructed stereoselectively using a glycosylation method developed in our laboratory. The synthetic route is rapid, efficient, and can easily be modified to make "mutant" oligosaccharides designed to test the importance of particular structural features in binding and specificity.

We have carried out 1D and 2D NMR experiments on the calicheamicin oligosaccharide and model systems for portions of it. We are using the NMR constraints in conjunction with molecular modelling to define the solution conformation of calicheamicin. We have had to modify the forcefield in the modelling package we are using to include parameters for the N-O bond and partial charges for the unusual sugars in calicheamicin. Parameters for the N-O bond were obtained from high level ab initio calculations and experimental data on simple substituted hydroxylamines.

We found a way to selectively hydrolyze the hydroxylamine linkage in calicheamicin  $\gamma 1$  without triggering the rearrangement. The new compound, calicheamicin T, lacks the B-C-D rings of calicheamicin. We evaluated the cleavage activity of calicheamicin T and found a) that it cuts DNA in a double-stranded manner like the parent compound, and b) that it has no significant cleavage specificity. In the course of this work, we also found that the cleavage specificity of calicheamicin is somewhat different than originally reported. For example, calicheamicin cuts TTTT sequences very effectively. Previous models for how calicheamicin recognizes DNA had included some key hydrogen bonds between guanine amino groups in the minor groove and the thiobenzoate ring in calicheamicin. Our work strongly suggests that calicheamicin recognizes a particular conformation of DNA and not a particular sequence.



SIGNIFICANCE: Sugars are components of many molecules that bind very tightly to DNA, but the role that the sugars play in these molecules has never been seriously investigated. We now have evidence that calicheamicin recognizes a particular conformation of DNA and not a particular sequence, and that the carbohydrate tail plays a very important role in the recognition process. Once we understand the factors that are important in recognition, we may be able to design other carbohydrate-based molecules that bind in predictable ways to DNA. This work could lead to the development of probes for DNA conformation.

WORK PLAN: The objectives next year are to:

1) Determine the binding energy and specificity of the calicheamicin oligosaccharide that we have synthesized in order to assess its contribution to the observed specificity of calicheamicin.

2) Synthesize key "mutant" oligosaccharides in order to test the importance of particular structural features in binding/cleavage activity. We are currently making an oligosaccharide in which the N-O linkage is replaced by a C-O linkage.

3) Set up a DNA cleavage assay to evaluate the specificity and efficiency of the mutant oligosaccharides. We will attach a non-specific cleaving agent to the calicheamicin oligosaccharide and the mutant oligosaccharides and compare the cleavage results in order to evaluate changes in binding energy and specificity.

4) Complete modelling studies to define the solution conformation of the calicheamicin oligosaccharide.

5) Undertake NMR studies on a calicheamicin-DNA complex.

INVENTIONS (last 12 months): one invention - calicheamicin

PUBLICATIONS AND REPORTS (last 12 months):

1. *Sugars as DNA Binders: A Comment on the Calicheamicin Oligosaccharide.*, S. Walker, K.G. Valentine, and D. Kahne\*, *J. Am. Chem. Soc.*, **1990**, 112, 6428.

2. *Construction of Glycosidic N-C linkages in Oligosaccharides*, D. Yang, S.H. Kim., and D. Kahne\*, *J. Am. Chem. Soc.*, **1991**, 113, 4715.

3. *Conformational Analysis of the N-O Bond in the calicheamicin Oligosaccharide*, S. Walker, D. Yang, D. Gange, and D. Kahne\*, *J. Am. Chem. Soc.*, **1991**, 113, 4716.

4. *On the origins of the Cleavage Specificity of Calicheamicin  $\gamma^1$* , S. Walker, R. Landovitz, W.-D. Ding, G. Ellestad and D. Kahne\*, submitted.

# ANNUAL PROGRESS REPORT

GRANT#: N00014-91-J-1231

R&T CODE: 441S012

PRINCIPLE INVESTIGATOR: Henry A. Lester

INSTITUTION: California Institute of Technology

GRANT TITLE: Ion Channel Blockade: A Sensitive Assay for Divalent Cations

REPORTING PERIOD: 1 November 1990 - 31 May 1991 (7 months)

AWARD PERIOD: 1 November 1990 - 31 October 1993

OBJECTIVE: To explore new methods for the heterologous expression of ion channels, with the ultimate goal of understanding the mechanism of ion channel blockade by divalent chimera.

ACCOMPLISHMENTS (last 12 months): Our work during this period diverged slightly from the goals of ion channel blockade, because we exploited a highly efficient new tactic for expressing ion channels in previously nonexcitable mammalian cells. Using the vaccinia/T7 system, we found that it was possible to express both electrically excitable potassium and sodium channels in the same cell. This gave us the opportunity to construct artificial neurons - that is, cells that fire neurons like action potentials in response to current stimuli. The artificial neurons provide a new method for evaluating the role of a particular ion channel in the encoding properties of neurons. In particular, we found that sustained repetitive firing could be obtained with only the rat brain IIA sodium channel and the *Drosophila* Shaker H4 potassium channel. We are now performing numerical simulations, in collaboration with Christof Koch and his colleagues at Caltech, in an effort to model the voltage trajectories of these cells.

SIGNIFICANCE: The construction of an artificial neuron holds promise for understanding the details of how a neuron encodes its firing frequency as a function of the stimuli that it receives. Such cells may also reduce the dependence on vertebrate animals in neuroscience research.

WORK PLAN (next 12 months): Now that the efficiency of the vaccinia system has been established for expressing ion channels, we are returning to the goal of structure-function relations in the permeability pathway of voltage dependent channels. Various chimeric channels are being constructed using PCR techniques from clones for calcium and potassium channels. The hope is to localize the permeation pathway to just a few amino acids in the putative  $\beta$  strands between transmembrane helices 5 and 6. In short, we would like to

transform a potassium sensitive channel into a calcium sensitive one.

As the various chimeric channels are constructed, they will be expressed in mammalian cells or in *Xenopus* oocytes and their permeation properties studied. We are very pleased with progress in other laboratories over the last year and we expect that insights will be coming at a great rate.

PUBLICATIONS AND REPORTS (last 7 months):

1. We are preparing a paper entitled "Neuronlike action potentials in mammalian cells after heterologous expression of voltage-gated channels."

2. We have submitted an abstract entitled "Why build artificial neurons?" to the Airlie House Meeting of the Biophysical Society on Biophysics and Recombinant DNA. The abstract is basically the same as the highlight page that follows.

PATENTS: Our method of constructing artificial neurons might be patentable. Do you think that the ONR could give us an opinion in this matter?

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1704

R & T CODE: 441s003

PRINCIPAL INVESTIGATOR: Douglas Prasher

INSTITUTION: Woods Hole Oceanographic Institution, Woods Hole, MA

GRANT TITLE: Genetic Engineering of Aequorin: Development of a Novel Metal Detection System.

REPORTING PERIOD: 21 June 1990 - 2 June 1991

AWARD PERIOD: 15 March 1990 - 14 March 1993

**OBJECTIVE:** The calcium-binding (CAB) domains in the photoprotein aequorin are being engineered to change the metal specificity of the luminescence.

**ACCOMPLISHMENTS (last 12 months):** Recombinant forms encoded by our three aequorin cDNAs AEQ1, AEQ2, and AEQ3, are now available; only recombinant AEQ1 and AEQ3 (rAEQ1 and rAEQ3) were available prior to this reporting period. Recombinant AEQ1 differs from AEQ2 and AEQ3 at 19 and 16 amino acid residues, respectively. Following charging (with luciferin), the three recombinant forms have been analyzed with regard to their total light yield (i.e. saturating calcium). Recombinant AEQ1 and 3 have full activity ( $> 10^{16}$  hv/mg) where rAEQ2 has less than 1% of their activity. Since rAEQ2 and rAEQ3 differ by only three amino acid residues, their significance in the 100-fold activity difference will be investigated.

A site-directed mutagenesis protocol (Lewis & Thompson, 1990, NAR 18:3439) is now being used to produce recombinant forms of aequorin. These modified aequorins are part of the project's first phase which is organized to address the role of each of the three calcium-binding domains in the luminescent reaction. Total light yield has been determined for four mutant aequorins having amino acid replacements designed to decrease the affinity for calcium. Three mutants have replacements in the first CAB domain (D31S, G36A, E42S) and the fourth has one in the second CAB domain (G129A). All four mutant proteins exhibit 5% or less the activity of wild type recombinant aequorin (rAEQ3). Expression vectors for several other mutant aequorins are at various stages of construction.

A pronounced calcium-independent luminescent activity (CIA) has been discovered at high pH, catalyzed not only by aequorin but also apoaequorin. Blinks et al. (Science 195:996, 1977) described a very weak calcium-independent activity at pH 7.0 catalyzed by native aequorin. The luminescence we observe at the high pH is much more intense. The CIAs for apo-rAEQ1, 2, and 3 parallels their total light yields of their charged counterparts, i.e. apo-rAEQ2 exhibits approximately 1% of the CIA of apo-rAEQ1 and 3. The four mutant forms of aequorin we have generated in vitro also show reduced CIA (1-5%) of the wild type rAEQ3. These results are consistent with the hypothesis that the CIA is due to a spontaneous conformational change leading to oxidation of the chromophore. The reduced activity of the mutant apoaequorins also suggest the luminescent mechanism is somehow related to the calcium dependent activity even though the light emission is supposed to be independent of calcium binding. The nature of these preliminary results prevents us yet from drawing too many conclusions but will be characterized further during the next year.

The purification of the recombinant aequorins is constantly undergoing refinement. The extraction procedure from the *E.coli* cells has been improved to increase the yield and quality (i.e. maximum total light yield) of the recombinant proteins.

Bruce Branchini (subcontract at Connecticut College) is now supplying synthetic benzyl luciferin to both Woods Hole and Linda Hannick (Naval Research Laboratory) who is performing the X-ray crystallography on the recombinant aequorin.

**SIGNIFICANCE:** The successful use of the mutagenesis protocol is now enabling us to address the functional roles of the three calcium binding domains in aequorin.

The discovery of luminescence catalyzed by apoaequorin can be used to compare the aequorin mutants and can potentially be useful in deciphering the mechanism of the calcium dependent luminescence.

The successful synthesis of luciferin will now enable Bruce Branchini to develop syntheses of luciferin derivatives designed to address the mechanism of aequorin's luminescence.

**WORK PLAN (next 12 months):** A limited number of additional aequorin mutants will be generated to complete the analysis of the three metal-binding domains. Then a variety of mutants will be generated which will contain amino acid replacements in one of the domains engineered to bind other metals more effectively than the wild type protein. Efforts will be taken to maintain the dependence of the luminescence on metal-binding.

New plasmid vectors will be constructed for improved efficiency of mutagenesis, expression, and purification. First, our current protocol uses separate vectors for mutagenesis and expression. A new vector will be constructed having features enabling both processes to be performed in a single vector. Second, the aequorin gene will be incorporated into a 'secretion expression vector' to explore improved production of correctly folded apoaequorin.

Characterization of the mutant aequorins will be expanded to include a variety of measurements designed to compare the aequorin derivatives. These include calcium-binding studies and lanthanide luminescence, charging efficiencies, absorption spectra, and circular dichroism spectra.

We will continue to consult with Linda Hannick on purification and crystallization of the recombinant protein and Bruce Branchini who will synthesize a new luciferin derivative designed to bind covalently (via a photoaffinity label) at the luciferin-binding site on the aequorin polypeptide.

**PUBLICATIONS AND REPORTS (last 12 months):**

1. DC Prasher, VK Eckenrode, WW Ward, FG Prendergast, and MJ Cormier. Primary structure of the *Aequorea* green-fluorescent protein. Submitted to *Gene*.
2. CC Cody, DC Prasher, WM Westler, FG Prendergast, and WW Ward. Sequence and chemical structure of the hexapeptide chromophore of the *Aequorea* green-fluorescent protein. Submitted to *J. Biol. Chem.*
3. DJ O'Kane, B Woodward, J Lee, and DC Prasher (1991) Borrowed proteins in bacterial bioluminescence. *Proc Natl Acad Sci USA* 88:1100.
4. DC Prasher, DJ O'Kane, J Lee, and B Woodward (1990). The lumazine protein gene in *Photobacterium phosphoreum* is linked to the *lux* operon. *Nucl Acids Res* 18:6450.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1986

R&T CODE: 441s009

PRINCIPAL INVESTIGATOR: Prof. K. N. Raymond, Prof. C. J. Hawkins,  
and Dr. L. R. Gahan

INSTITUTIONS: University of California, Berkeley, and  
University of Queensland, Australia

GRANT TITLE: Piezoelectric Metal Biosensors

REPORTING PERIOD: 1 April 1990 - 30 June 1991

AWARD PERIOD: 1 April 1990 - 31 March 1993

OBJECTIVES: To investigate:

1. the effects of solutions on the vibration frequency of quartz piezoelectric transducers;
2. the covalent attachment of ligands with designs based on marine metal sequestering agents to the surface of quartz piezoelectric crystals;
3. the surface modification of quartz crystals using polymeric materials, particularly those that would enhance the selective binding of cations;
4. the effects of group IIB and d transition metal ions on the vibration frequency of modified quartz crystals; and
5. the application of surface-modified quartz piezoelectric transducers as analytical devices for the detection of transition metal ions in oceanic waters.

ACCOMPLISHMENTS - University of Queensland:

- 1) The frequency change response of 10 MHz AT-cut Ag-electroded piezoelectric crystals to aqueous NaCl (concentration 0.5 to 1.0 mM) is linear in this region. Our results with LiCl, NaCl, KCl, RbCl, and CsCl suggest that the response of the crystal may be a function of the size of the hydrated cation.
- 2) We have begun the synthesis of cation selective receptor species suitable for attachment to the surface of a piezoelectric crystal. We have prepared three macrocyclic precursors which possess a secondary amine group suitable for further synthetic elaboration. The attachment of long alkyl chains ( $C_{22}H_{45}-$ ) has been achieved for two of these macrocycles and studies to investigate the deposition of thin films (monolayers) of the receptors using Langmuir-Blodgett techniques are in progress. Silane derivatives of these macrocycles have been prepared by attaching  $C_{16}$  chains possessing a pendent  $-HC=CH_2$  moiety. The alkane can be readily hydrosilated to give a terminal  $-Si(OEt)_3$  moiety suitable for reaction with the crystal surface. The result will be a covalently attached macrocyclic species.
- 3) Our work on the isolation and identification of cyclic peptides and catecholate chelators from marine organisms continues. We are interested in the presence in the oceans of metal complexes of biologically active cyclic peptides from the didemnid ascidians. ICP-MS has been used to study 13 aplousobranchs to see which metals, beside iron and vanadium, are accumulated, and to see if there is a relationship between the metals and the type of chelates isolated from the animals. A number of hydroxylated compounds have been isolated from ascidians, some of which are proteins. These compounds are strong chelators of metal ions; structural investigations are underway.
- 4) We are continuing to pursue the design of the crystal devices. Recently, we have commenced an investigation of "electrodeless piezoelectric devices" following a report by Nomura et. al. (*Anal. Chim. Acta.* 1991, 243, 273-8). Our previous studies have highlighted the sensitivity of the silver electrodes to chemical disruption. The

"electrodeless" piezoelectric crystals offer the possibility of using a device with no attached electrode. We have built a device in which a blank 10MHz quartz plate is attached to a glass cylinder of the same diameter. The cylinder is filled with electrolyte solution (0.1 M KCl) and a platinum electrode immersed in the solution. Attached to the outside of the cylinder is another platinum electrode. Attachment of the oscillator circuit and immersion of the device in electrolyte solution results in the oscillation of the crystal (~9.9 MHz). The device is robust and simple.

ACCOMPLISHMENTS - University of California:

We are currently investigating ways to attach catecholate-based metal-specific ligands to  $\text{SiO}_2$ . As part of a related project, we are designing octadentate actinide sequestering agents for attachment to silica. We have made some progress in functionalizing the silica, and some of this chemistry can be utilized for attachment of these ligands to quartz surfaces. We have recently begun the synthesis of catecholate ligands that can be attached to  $\text{SiO}_2$  in a variety of ways.

In May 1991 Dr. Peter Bonnesen (Berkeley) spent three weeks in Dr. Gahan's laboratory at the University of Queensland working on both the silver electroded and "electrodeless" crystals in solution. The design of "electrodeless" crystal device has been improved and a crystal oscillator setup is being constructed at Berkeley, so that the catecholate derivatized crystals may be tested directly.

SIGNIFICANCE: We are producing cation-selective receptors for attachment to piezoelectric crystals in resonating circuits. The compounds are modeled on those observed in biological systems. Our "electrodeless" crystals point to the production of a robust, almost chemically inert, device which operates in the presence of a background electrolyte, perhaps akin to the conditions in seawater. Ligand synthesis for metal selective complexing agents is proceeding well both at Berkeley and at Queensland. The ability to selectively target metal ions using a matrix array remains a significant, if still distant, goal.

WORK PLAN: Over the next 12 months we will pursue all aspects of the project as proposed initially.

PUBLICATIONS AND REPORTS: none

## **MOLECULAR BIOLOGY OF MARINE ORGANISMS**

### **CORE PROGRAMS**

**SCIENTIFIC OFFICERS: DRS. RANDALL S. ALBERTE AND ERIC EISENSTADT**

**PROGRAM OBJECTIVES: TO DETERMINE THE BASIC BIOLOGICAL MECHANISMS GOVERNING THE PHYSIOLOGICAL ACTIVITIES OF MARINE MICROBES (BACTERIA, FUNGI) AND ALGAE AND TO DEVELOP NOVEL MOLECULAR APPROACHES FOR ANALYZING THE PHYSIOLOGY OF NATURAL POPULATIONS OF MARINE ORGANISMS.**

**NAVY OBJECTIVE: TO DEVELOP NEW BIOTECHNOLOGIES BASED ON THE UNIQUE CAPABILITIES OF MARINE ORGANISMS THAT CAN BE APPLIED TOWARDS THE SOLUTION OF SUCH LONG TERM NAVAL NEEDS AS THE CONTROL OF BIOFOULING AND THE ELIMINATION OF HAZARDOUS SUBSTANCES IN MARINE ENVIRONMENTS.**



# ANNUAL PROGRESS REPORT

GRANT#: N00014-89-j-3154

R&T CODE: 441d025

PRINCIPAL INVESTIGATOR: M. Robert Belas

INSTITUTION: Center of Marine Biotechnology  
University of Maryland System

GRANT TITLE: The Molecular Mechanism of Sensory Transduction  
in a Marine *Vibrio*

REPORTING PERIOD: 1 July 1990 - 30 June 1991 (12 months)

AWARD PERIOD: 1 July 1989 - 30 June 1992

OBJECTIVE: It is the goal of this laboratory to understand how environmental signals control gene expression in marine bacteria. The model system we have chosen to explore is the surface-induced regulation of *Vibrio parahaemolyticus* swarmer cell differentiation. The specific aim of the proposed research is to focus on the genes and the regulatory proteins they encode which control expression of *laf* genes.

ACCOMPLISHMENTS (last 12 months): We have concentrated on two general classes of mutants which give rise to abnormal regulation of *laf* genes. The first class of mutants is composed of those strains which have defects in chemotactic behavior (*Che*<sup>-</sup>). In our first year of funding, we isolated 15 *Che*<sup>-</sup> mutants, characterized them as to the specific nature of each defect, and cloned out a DNA fragment corresponding to part of each *che* gene. Seven of the 15 *Che*<sup>-</sup> mutants produced swarmer cell in (normally) non-inducing conditions. During the past year, we have identified the intact *che* genes in a cosmid library composed of large inserts of *V. parahaemolyticus* genomic DNA. Linkage analysis reveals that at least three of the genes appear to be genetically linked. We are in the process of subcloning each *che* gene and identifying the 5' and 3' ends.

The second class of mutants defective in the normal surface-induced regulation of *laf* genes was discovered by mutagenizing BB1301 (*laf::lux* 1301, Tet<sup>r</sup>, Rif<sup>r</sup>), a strain carrying a surface-inducible luciferase transcriptional fusion. Following mutagenesis with mini Tn5 Sp/Sm, we examined 8,300 spectinomycin-resistant colonies for abnormal regulation of light. This screening was greatly simplified by using a multiwell, microtiter plate luminometer (Dynatech, model ML1000). Of the 8,300 original mutants, 72 were found to abnormally regulate expression of the *laf::lux* fusion. Two groups of mutants were found: Class I, constitutive "on", and Class II constitutive "off". Those mutants defined as constitutive "on" demonstrated elevated levels of luciferase activity well above wild-type in both inducing and noninducing conditions. Constitutive "off" mutants on the other hand were defined as those strains which expressed very low levels of luciferase at all times and were "blind" to the inducing stimulus. Since defects in polar flagellum rotation and chemotaxis have been shown

to cause abnormal control of swarmer cell differentiation, the 72 strains were tested for swimming motility using a semi-solid agar medium. Approximately 40% of these strains were either Fla<sup>-</sup>, Mot<sup>-</sup>, or Che<sup>-</sup> and not used in further analyses. The remaining 53 mutants were wild-type in all aspects except that they were defective in the normal surface-induced control of swarmer cell differentiation. Interestingly, in each class of mutants, there were groups of strains which possessed nearly identical light production, yet had the transposon inserted at different sites. This grouping of strains within Class I and Class II mutant categories suggests that several genetic loci are involved in the regulation of *laf* genes.

**SIGNIFICANCE:** First, we interpret these data to mean that there are in fact multiple regulatory genes (perhaps acting in a cascade of regulation) which control the expression of *laf::lux*. This is supported by our data demonstrating both constitutive "on" and constitutive "off" classes of mutants. Further, it also suggests that both negative and positive regulation must be involved in the control of swarmer cell differentiation, not just negative regulation as has been suggested by others. Additionally, multiple regulatory genes could explain the apparent grouping of mutants in each class. For example, in the Class I (constitutive "on") at least two types of mutants are evident based upon the percentage of luminescence in uninduced cells when compared to wild-type. In this class, there are mutants which show (1) greater than 1,000% but less than 10,000% light production, and (2) those which produce greater than 10,000% light when compared to wild-type. Furthermore, some mutants (although called constitutive) do show a slight surface induction. We interpret this to indicate that even though these mutants are defective in one aspect of the wild-type regulation, secondary and tertiary regulators may act to control the *laf::lux* fusion.

**WORK PLAN (next 12 months):** We plan to continue our efforts to analyze the Che<sup>-</sup> mutants and to delineate the nature of the defect as it pertains to *laf* regulation. Moreover, we believe that our data support the idea of multiple regulatory genes involved in the control of swarmer cell differentiation. We will thus focus our efforts on elucidating the nature of the mutations in both the Class I and II mutants. Of particular interest to us now are two goals: (1) How many regulatory genes have we defined in our two classes, and (2) What is the regulatory hierarchy (if one exists) between the different regulatory genes? Lastly, we plan to place the mutated regulatory genes back into wild-type vibrio and examine their effect. It is feasible that the various regulatory genes function to control specific aspects of the differentiation process.

**INVENTIONS:** None.

**PUBLICATIONS AND REPORTS (last 12 months):** R. Chien and R. Belas. 1991. Multiple regulatory genes control *Vibrio parahaemolyticus* swarmer cell differentiation. In review.

# ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1917

R&T CODE: 400x073yip

PRINCIPAL INVESTIGATOR: Edward F. DeLong

INSTITUTION: Woods Hole Oceanographic Institution

GRANT TITLE: Phylogenetic analysis of bacterial assemblages associated with marine macroaggregates.

AWARD PERIOD: 01 June 1990 - 30 May 1993

OBJECTIVE: To determine, by use of molecular probes for rRNA sequences, the species and functional diversities of marine bacterial populations inhabiting aggregates, as well as planktonic bacterial populations living unattached in the water column.

ACCOMPLISHMENTS: Initial work included completion of a WHOI research diving course by the PI, in August 1990. Subsequently, planktonic "free-living" bacterial populations, as well as microbial assemblages associated with macroaggregates, were collected in the Santa Barbara channel during October 9-12, 1990, and June 4-6 1991 (in a collaborative effort with Dr. Alice Alldredge's group at UC Santa Barbara). Although on a few collecting days aggregates were scarce, on most days we were able to hand collect hundreds of aggregates on each dive. Seawater concentration involved in situ pre-filtration of 40 liters of seawater by divers, followed by hollow fiber filtration and high speed centrifugation, resulting in high recovery (80-95%) of planktonic bacterial forms. Macroaggregates were hand collected by divers in sterile syringes.

DNA has been isolated from both the aggregate and "free-living" bacterial fractions, for molecular phylogenetic characterization of population constituents. Yields of nucleic acids from the aggregates ranged in the tens of micrograms, therefore the polymerase chain reaction (PCR) was employed to amplify small subunit ribosomal RNA (rRNA) genes from the mixed population DNA. Amplified rRNA genes were then cloned into recombinant plasmid vectors for subsequent sequencing and phylogenetic analyses. Aggregate and "free-living" microbial population PCR libraries were constructed, using sets of amplification primers broadly specific for archaeobacteria, eubacteria and eukaryotes. Unique clones in each of these libraries are now being sequenced and compared. These sequences are being used to design rRNA probes specific for particular groups. One exciting finding has been our

recent discovery of novel types of archaebacteria, which appear most closely related to methanogens, associated with the aggregates (see Highlight Page). In attempts to isolate these micrororganisms in pure culture, we have subsequently retrieved sulfate reducing bacteria in culture, using macroaggregates as inocula. These combined data are provocative, and consistent with the notion that aggregates may serve as sites for anaerobic microbial activities, in an otherwise aerobic water column.

SIGNIFICANCE: Rapidly sinking, macroscopic aggregates are largely responsible for the flux of organic matter to the deep-sea. Associated microbial populations likely participate in many aggregate-mediated processes, including primary production, decomposition, and aggregate formation and diagenesis. Our study is revealing the community structure of these attached bacterial assemblages, whose physiological and phylogenetic diversity are largely unknown. Knowledge of the diversity and variability of attached microbes will provide insight into the influence of microorganisms on the physical properties, nutrient quality, and vertical transport of macroaggregates in the sea.

WORK PLAN: The next year will be spent characterizing clones obtained in our initial collections, and designing probes specific for particularly interesting or abundant types. We will use radioactively-labeled probes to quantify specific groups in aggregates and seawater. We will also further develop methods for using fluorescently labeled probes with epifluorescence microscopy and flow cytometry, to quantify specific population members. Collection of aggregates from other geographic locales will also be attempted.

PUBLICATIONS and REPORTS:

1. D. L. Distel, E. F. DeLong and J. B. Waterbury. 1991. Phylogenetic characterization and in situ localization of the bacterial symbiont of shipworms, using 16S rRNA sequence analysis and oligodeoxynucleotide probe hybridization. Appl. Environ. Microbiol., in press, August 1991 (galley enclosed).
2. Schmidt, T. M., E. F. DeLong, and N.R. Pace. 1991. Analysis of a marine picoplankton community using 16S rRNA gene cloning and sequencing, J. Bact., in press, August 1991. (preprint enclosed)
3. DeLong E. F. 1991. Molecular systematics, microbial ecology and single cell analysis. In S. Demerge, ed., Single Cell and Particle Analysis in Oceanography, NATO Advanced Study Series, Springer Verlag. (preprint enclosed)
4. DeLong, E. F., 1991. Aquatic habitats: High Pressure Environments, In J. Lederberg ed., Encyclo. Microbiol., Acad. Press (preprint enclosed)

Grant#: N00014-91-J1290

R&T Code: 4412 094---01

PRINCIPAL INVESTIGATOR: Richard B. Frankel

INSTITUTION: California Polytechnic State University  
San Luis Obispo, CA

GRANT TITLE: Biomineralization of Fe Oxides and Sulfides in  
Magnetotactic Bacteria

REPORTING PERIOD: 1 January 1991 - 30 June 1991 (6 months)

AWARD PERIOD: 1 January 1991 - 31 December 1993

OBJECTIVE: To investigate magnetosome structure and biomineralization processes in iron oxide and sulfide producing magnetotactic bacteria. Co-principal investigators include Dennis Bazylinski at Virginia Polytechnic Institute and State University and Anthony Garratt-Reed at MIT.

ACCOMPLISHMENTS (last six months):

a) We have determined the morphologies of greigite ( $\text{Fe}_3\text{S}_4$ ) particles from two different magnetotactic rods found in marine sulfidic environments using electron diffraction and high-resolution electron microscopy. We find cubo-octahedral particles in one organism and rectangular-prismatic particles in the other. Greigite is the only mineral found in these bacteria, in contrast to a large multicellular bacterium from the same environments that produces both greigite and pyrite ( $\text{FeS}_2$ ). This result demonstrates that iron sulfide production in these bacteria is as precisely controlled as is biomineralization in magnetite ( $\text{Fe}_3\text{O}_4$ ) producing bacteria. It also suggests that the large multicellular organism is separately mineralizing greigite and pyrite in the same cell.

b) We have devised a method for separation and accumulation of large numbers of magnetotactic bacteria from sediments and other microorganisms that utilizes a modification of the R. Wolfe "racetrack" technique. This has facilitated investigation of wild type bacteria and accumulation of inocula for culturing studies.

c) We have used the technique to prepare samples of the large multicellular bacterium for phylogenetic studies in collaboration with E. Delong at Woods Hole Oceanographic Institution. Ribosomal RNA from this organism has been cloned and in addition, amplified using PCR technology. 16S rRNA sequencing is in progress. Preliminary results suggest that the multicellular organism is not closely related to the magnetite producing magnetotactic bacteria.

d) Three and possibly more new strains of magnetotactic bacter have been isolated and cultured using oxygen

gradient media. Energy dispersive x-ray analysis shows that all these strains are magnetite producers. One organism, a coccus with two flagellar tufts, accumulates large polyphosphate bodies and sulfur granules when grown chemolithotrophically. When grown heterotrophically, the organism does not produce sulfur granules but does accumulate small polyphosphate granules. Studies of microbial physiology are underway to boost cell yields for magnetosome and genetic analysis. We have also devised techniques for breaking open cells and extracting magnetosomes from the cell debris.

e) We have constructed a facility for precise control of the ambient magnetic field direction and magnitude over a volume of one square meter. This facility will allow determination of the permanent magnetic dipole moments of individual bacteria, and the variation of the moment with conditions of culture.

SIGNIFICANCE: The isolation and culture of new strains of magnetotactic bacteria will allow us to carry out comparative studies of magnetosome structure which should provide clues to biomineralization processes in the magnetite producing bacteria. Our expectation is that phylogenetic analysis will provide physiological clues and allow for the isolation and culture of strains of iron-sulfide producing bacteria for magnetosome structure and biomineralization studies.

WORK PLAN (next twelve months): Our primary objective in the next twelve months is to analyse the magnetosome membranes for the magnetotactic bacteria strains which are currently in pure culture. This will be done both by electron microscopy of stained thin sections, as well as by magnetic extraction of intact magnetosomes from broken cells, and chemical analysis of the membrane components. In addition, we will use gel electrophoresis to characterize membrane and cytoplasmic protein fractions. We will complete the phylogenetic analysis of the large multicellular bacterium and continue our efforts at isolation and culture of new strains of iron-oxide and iron-sulfide producing bacteria.

PUBLICATIONS AND REPORTS (last six months):

1. D.A. Bazylinski: Bacterial Production of Iron Sulfides, in: *Materials Synthesis Based on Biological Processes* Vol. 218, Eds. Alpert et al (Materials Research Society, Pittsburgh, PA) in press, 1991.

2. B.R. Heywood, S. Mann, and R.B. Frankel: Structure, Morphology and Growth of Biogenic Greigite ( $\text{Fe}_3\text{S}_4$ ), in: *Materials Syntheses Based on Biological Processes* Vol. 218, Eds. Alpert et al (Materials Research Society, Pittsburgh, PA) in press, 1991.

ANNUAL PROGRESS REPORT

GRANT #: N00014-88-0352

R&T CODE: 4412048

PRINCIPAL INVESTIGATOR: Elma Gonzalez

CO-PRINCIPAL INVESTIGATOR: David J. Chapman

INSTITUTION: University of California at Los Angeles

GRANT TITLE: Molecular Mechanisms in Cell Wall Assembly and  
Calcification of Marine Phytoplankton

REPORTING PERIOD: 1 July 1990 - 30 June 1991

AWARD PERIOD: 1 July 1988 - 31 December 1991

RESEARCH OBJECTIVE: To investigate the molecular and cellular basis for cell wall calcification in coccolithophorids.

ACCOMPLISHMENTS (last 36 months): We carried out basic investigations designed to reveal optimal conditions for growth and mineralization of 3 candidate organisms; *Coccolithus pelagicus*, *Hymenomonas carterae*, and *Pleurochrysis scherffelli*. Both synthetic and semi-synthetic growth media have been tested and parameters such as generation times, and the percentage of cells exhibiting calcified coccoliths have been monitored using light and scanning electron microscopy. The influence of environmental factors such as light intensity, aeration, and buffering capacity of the growth medium, on coccolithogenesis have also been examined.

We also undertook a detailed ultrastructural analysis of *Coccolithus pelagicus* to determine the effect of inorganic sulfate depletion on coccolithogenesis. The abundance of mineralized coccoliths and the fidelity of their morphology was monitored by means of scanning electron microscopy. The cultures grown on completely synthetic medium containing inorganic sulfate concentrations ranging from 60 to 30,000 micromolar were also examined by transmission electron microscopy. We observed severe damage to cellular ultra-structure at the lowest levels of sulfate. However, we did not observe an impact on the normal morphology of coccoliths that could be explained by the overall derangement of cellular integrity caused by low sulfate. On the basis of these results we tentatively conclude that the templating mechanism underlying morphological fidelity of crystallized coccoliths is not sensitive to low sulfate in the growth medium.

We have continued to develop a molecular approach to calcification using *Coccolithus pelagicus*. During the first year we were able to carry out successful isolations of genomic DNA sufficiently clean for restriction enzyme analyses. We have also successfully isolated and purified poly (A)<sup>+</sup> RNA which we have also successfully translated in vitro.

We have also made significant progress in adapting and developing techniques for subcellular characterization of mineralizing *Coccolithus* cells. We have resolved Golgi membranes on density gradients. We have demonstrated, for the first time, the presence of generally accepted Golgi markers, such as inosine diphosphatase and cation-dependent ATPases, in sub-cellular fractions of a marine, unicellular alga.

We have now established the existence of two Golgi compartments in homogenates of *C. pelagicus*. A "light Golgi" with a density (1.127 g/cc), which is typical of higher plant Golgi, and a "heavy Golgi" with a density (1.156 g/cc) both contain UDPase. The "heavy Golgi" compartment appears to be further resolvable into membrane fractions

enriched for K<sup>+</sup>-stimulated, Mg<sup>2+</sup>-ATPase, and Ca<sup>2+</sup>-ATPase. Calcium in the form of Calcium carbonate is primarily associated with the "heavy" Golgi membranes most enriched for Ca<sup>2+</sup>-ATPase. The ion transporting capacities of the various membrane fractions and their co-factor requirements are now being examined.

Other progress: In 1989 University funds were secured which permitted us to redesign and remodel our antiquated culture facility. We now have more space for cultures. We also have improved electrical outlets and amperage, light fixtures, temperature and compressed air controls.

SIGNIFICANCE: A molecular/cellular approach to important questions regarding the mechanisms of calcification, secretion and cell wall assembly in marine algae is made possible for the first time as we continue to develop *Coccolithus* as an experimental system with unique potential.

WORK PLAN (next 12 months): Our objective next year is to continue our studies on Golgi. We specifically want to accomplish maximum organelle enrichment and thorough characterization of the membranes in terms of their enzymatic activities and their respective polypeptide complements. We would like to distinguish between the cis and trans Golgi apparatus. Since we know that mineralization takes place in the trans membranes or vesicles, we are eager to proceed to a characterization and isolation of polypeptides from those membranes. Our second objective is to construct an expression library of DNAs complementary to mRNAs expressed during coccolithogenesis.

#### PUBLICATIONS AND REPORTS:

Wainwright, I.M., D.-K. Kwon, and E. Gonzalez. 1991. Intracellular calcification in *Coccolithus pelagicus* (Copel): isolation and characterization of Golgi. *J. Phycol.* 27 (3): S75.

Wainwright, I.M., D.-K., Kwon, and E. Gonzalez. 1991. Isolation and characterization of Golgi from *Coccolithus pelagicus*. *Plant Physiol.* 96 (1): S131.



## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-3027

R&T CODE: 441d026

PRINCIPAL INVESTIGATOR: Govind S. Nadathur

INSTITUTION: The University of California at Santa Barbara

GRANT TITLE: *Debaryomyces hansenii*: A Model System for  
Marine Molecular Biology

REPORT PERIOD: 1 June 1990 - 30 May 1991

AWARD PERIOD: 1 June 1989 - 31 December 1991

OBJECTIVE: To establish *Debaryomyces hansenii* as a model for marine molecular biology. Specifically, the objective is to develop plasmid vectors, a transformation system and have a good number of mutants available in this organism.

### ACCOMPLISHMENTS:

#### 1. Isolation of Autonomously Replicating Sequences (ARS) From *Debaryomyces*:

In an attempt to construct plasmids with the capability of autonomous replication we have isolated two ARS from *Debaryomyces* utilizing *S. cerevisiae* strain SEY 2108 (*Ura3*<sup>-</sup>) and its corresponding gene *URA3* as a marker. The first, a 400bp. insert (pAB81) was selected for further analysis. The ARS activity was found to reside in 150bp. of the clone (pAB83). Sequencing of this region revealed the existence of an AT rich region with the consensus sequence TTTATRTTT, which is known to be present in all sequences known to function as ARS in yeast. Attempts are now being made to transfer *Ura3*<sup>-</sup> *Debaryomyces* with this plasmid.

Transformation attempted with techniques such as protoplasting, alkali cations and electroporation has not met with success. This may be an attribute of the strain being used. With the arrival of strain J-26 for Dr. L. Adler, transformation will be attempted utilizing hygromycin resistance as a marker. Dr. Adler has also agreed to generate *Ura3*<sup>-</sup> mutants of his strain for use as a marker.

#### 2. Mutagenesis of *D. Hansenii*:

To generate auxotrophic mutations for transformation, mutagenesis was performed utilizing Ethyl Methyl Sulfonate. *Ura3*<sup>-</sup> were selected by the negative selection technique utilizing 5-fluoro orotic acid. Even though this technique generates a large number of mutants, the stability of the mutants is questionable. Dr. L. Adler, University of Goteborg, Sweden has kindly agreed to send his strain of the organism which apparently has been very successful in mutant generation.

#### 3. Isolation of 17S rRNA and Ubiquitin Genes:

To determine the phylogenetic position of *Debaryomyces*

relative to *S. cerevisiae* and *Candida albicans* the 17S rRNA and Ubiquitin genes have been isolated with the help of specific oligonucleotide primers and polymerase chain reaction. The 17s rDNA has now been sequenced and found to have 1798 bp. The phylogenetic comparisons are now being performed.

## FINAL PROGRESS REPORT

GRANT #: N00014-87-K-0813

R&T CODE: 4112030

PRINCIPAL INVESTIGATOR: Norman R. Pace

INSTITUTION: Indiana University

GRANT TITLE: Phylogenetic Analysis of Marine Picoplankton using rRNA Sequences

REPORTING PERIOD: September 1987 - August 1991

AWARD PERIOD: September 1987 - September 1991

OBJECTIVE: To analyze phylogenetically the dominant constituents of oligotrophic marine picoplankton at selected sites using ribosomal RNA gene cloning and sequencing.

**Overview:** This report is a summary of the overall project period. Formal funding for the project ended 02/91, although a small component was extended until 9/91. The overall essential goals of the program during the main period of performance were outstanding. The essential goals of the program were fulfilled. The activities of the laboratory in natural population analysis during the project period have had good impact on the fields of microbial ecology and microbial phylogenetics.

### ACCOMPLISHMENTS:

*Phylogenetic identification of picoplankton.* Using tangential flow filtration, we collected bulk amounts of marine picoplankton from the central Atlantic Ocean (Sargasso Sea, Hydrostation S) and Northern Pacific Ocean (Aloha Station). DNA prepared from both populations was analyzed by hybridization using kingdom-specific probes complementary to 16S rRNA, and determined to be largely (>90%) eubacteria: little eukaryotic-specific and little or no archaeobacterial-specific DNAs were detected. DNA from the Aloha Station was suitable for cloning experiments and a random library of 20 kb *Sau3a* fragments in phage  $\lambda$  EMBL3 was established. This library is available to other investigators upon request. During these past two years, screening that library for 16S rRNA-containing clones, sequencing the genes and carrying out phylogenetic analysis occupied substantial effort. Approximately 50 rRNA gene-containing clones were identified by hybridization and the unique types determined by sequencing using single dideoxynucleotide reactions. Several hundred nucleotides of sequence was determined for each of the unique types.

The Aloha picoplankton proved to be a fairly simple population. Only 15 different types of rRNA genes have been identified, 14 eubacteria and one eukaryote (a dinoflagellate). The eubacteria are all restricted to well-known phylogenetic groups, the  $\alpha$ - and  $\gamma$ -groups of "proteobacteria" ("purple" bacteria, *sensu* Woese), and the cyanobacteria. The identified cyanobacterial picoplankton are all closely related to *Synechococcus* spp. Considerable information is known about the proteobacteria and the cyanobacteria phylogenetic groups that can be extrapolated to the picoplankton, since properties that are common to a phylogenetic group are expected to occur in any particular representative of that group.

*Quantitative analysis of the clones.* We are currently working towards a quantitative assay of cloned rRNA genes in mixed populations. This should approximate the distribution of the organisms in the population. We find, however, that simple hybridization tests using probes based on rRNA sequences are not optimal for quantitative analysis. Longer probes (>ca. 50 nucleotides) typically are problematic because of cross-species hybridization due to sequences shared among

related organisms. Oligonucleotide probes (<ca. 30 nucleotides) can discriminate between targets on the basis of only one or a few mismatches, but the stringency of hybridization required for such precision also results in loss of much or most of the homologous signal. (Theoretical reasons for this are based on the high dissociation rates of oligonucleotide hybrids). The necessity to achieve a balance between specificity and quantitation is difficult to control for, experimentally, and it casts uncertainty on many oligonucleotide hybridization results, particularly in the case of low-abundance sequences. Because of this uncertainty, we are developing a method for quantitative analysis of clone distribution based on a nuclease protection assay to discriminate perfectly matched hybrids mismatched ones. The method should be generally useful for studies in microbial ecology.

*Continued development of single-cell phylogenetic stains.* We previously showed that fluorescently labeled oligonucleotides complementary to phylogenetic group-specific sequences can be used to identify single cells. Probes used initially had a singular fluor molecule, fluorescein or rhodamine, attached to the oligonucleotide. Consequently, detection of probe-binding to individual cells requires the presence of ca. 5000 ribosomes (the probe targets). In some classes of organisms (e.g. phototrophs) probe-binding also is compromised by intrinsic fluorescence that overwhelms probe-fluorescence. A method is needed for bleaching cells that does not interfere with subsequent hybridized results. In order to increase the sensitivity of the fluorescent probes, we have experimented with increasing the number of fluor molecules attached to oligonucleotides. However, addition of multiple probes to one end of the oligonucleotide results in reduction of fluorescence (due to quenching); addition of fluors to multiple nucleotides in a given oligonucleotide results in destabilization of the hybrid. Nearly two-fold gain in sensitivity is achieved by attaching fluors at both 5'- and 3' ends of oligonucleotides. However, further sensitivity through adding multiple fluors will require use of longer probes so that fluor molecules can be sufficiently well-separated to minimize quenching.

*Use of PCR for population analysis.* A random recombinant library in a phage vector is the least selective method available for retrieving rRNA genes from a mixed population of organisms, but it requires substantial DNA (>100 µg) and, consequently, substantial biomass. In order to accommodate small amounts of natural DNA, we (and others) are using "polymerase chain reaction" (PCR), coupled with primers complementary to universally conserved rRNA sequences, to amplify rRNA genes for cloning and sequence analysis. We are interested in using micromanipulation to obtain cells from the environment for phylogenetic analysis. Substantial experience has now been gained with PCR. In general the method works well, but some noteworthy problems have been encountered.

A significant problem in the use of universally conserved rRNA sequences as PCR primers is that all commercially available Taq DNA polymerase is contaminated by organismal DNA. This contaminating DNA presumably derives mostly from chromatography columns, etc., used in preparing the enzyme. Although (usually) not a problem with other primers, the universally applicable rRNA primers detect the DNA and the resulting signal can eclipse that due to a low amount of added template. In an effort to reduce the contaminating DNA, we obtained an expression clone of Taq DNA polymerase and developed an enzyme purification protocol that reduces, but does not yet eliminate, contaminating DNA. In-house production of Taq polymerase has been economically useful, as well: we obtain >106 units of this expensive enzyme from 1 l. of induced cells. We are still working at suppressing residual contamination, but the improved purification method results in enzyme preferable for population analysis.

Another potential problem with PCR is that rRNA genes of different organisms in a mixed population may not amplify equally well. We have preliminary evidence that this may be a problem, and are currently investigating the issue.

**TRAINING:** During the course of the program the laboratory served as host to training a number of investigators from other institutions in the use of rRNA-based technology. These visitors recognized the utility of the methods to their own ecological or phylogenetic studies. Examples of visitors include F. Azaur (S.I.O.), T. Burger-Wiersma (U. Amsterdam), C. Cavanaugh (Harvard), D. Distel (S.I.O.), J. Fuhrman, R. Rossen (Center for great Lakes Studies), J. Waterbury (W.H.O.I.), and others.

**FUTURE DIRECTIONS:** The program to characterize picoplankton and develop rRNA-based microbiology methods has been successful. The work with picoplankton continues with former colleagues: E. DeLong (now at W.H.O.I.), S. Giovannoni (Oregon State), and T. Schmidt (U. Miami). Future efforts of the laboratory will continue with development of methods for population analysis without cultivation of target organisms. However, main focus will be on high-temperature ecosystems.

#### **PUBLICATIONS AND REPORTS:**

1. Pace, N.R. and A.B. Burgin (1990). Processing and evolution of the ribosomal RNAs. In "The Structure, Function and Evolution of Ribosomes," W. Hill (ed.). Am. Soc. Microbiol., pp. 417-425.
2. DeLong, E.F., T.M. Schmidt, and N.R. Pace. (1990) Analysis of single cells and oligotrophic picoplankton populations using 16S ribosomal RNA sequences. In "Recent Advances in Microbial Ecology," T. Hatori, Y. Ishida, Y. Maruyama, R. Morita, and A. Ucida (eds). Japan Scientific Societies Press, pp. 697-701.
3. Giovannoni, S.J., E.F. DeLong, T.M. Schmidt and N.R. Pace (1990). Tangential flow filtration and preliminary phylogenetic analysis of marine picoplankton. Appl. Environ. Microbiol., 56:2572-2575.
4. Schmidt, T.M., E.F. DeLong and N.R. Pace. (1991). Phylogenetic identification of uncultivated microorganisms in natural habitats. In "Rapid Methods and Automation in Microbiology and Immunology." Springer-Verlag, in press.
5. Eden, P.A., T.M. Schmidt, R.P. Blakemore, and N.R. Pace. (1991). Phylogenetic analysis of *Aquaspirillum magnetotacticum* using PCR-amplified 16S ribosomal RNA-specific DNA. Internat. J. System. Bacteriol. 41:324-325.
6. Schmidt, T.M., B. Pace and N.R. Pace. (1991). Detection of DNA contamination in Taq polymerase. BioTechniques (in press).
7. Schmidt, T.M., E.F. DeLong and N.R. Pace. (1991). Analysis of a marine picoplankton community using 16S rRNA gene cloning and sequencing. J. Bacteriol. 173:4371-4378.
8. Pace, N.R. (1991). Origin of life-Facing up to the physical setting. Cell 65:531-533.

## ANNUAL PROGRESS REPORT

GRANT #: -----

R&T CODE: 4412110---01

PRINCIPAL INVESTIGATOR: F. G. Plumley

INSTITUTION: University of Alaska

GRANT TITLE: Marine Diatom Plasmids and their Biotechnological Applications

REPORTING PERIOD: -----

AWARD PERIOD: 01 June 1991 - 31 May 1993

OBJECTIVE: The long-range objective of the proposed research is to achieve transformation of marine diatoms. The more immediate objectives pertain to the characterization of two small plasmids we have discovered in a marine diatom, Cylindrotheca fusiformis, a species which comprises 50% of the biofouling community on steel structures in marine environments.

Specific questions being addressed are:

- 1) What are the structures, functional and regulatory properties of the two diatom plasmids?
- 2) Are there sequences on these plasmids which will facilitate construction of diatom shuttle-cloning vectors?
- 3) What are the necessary conditions for achieving diatom transformation?

ACCOMPLISHMENTS: This is a new grant.

SIGNIFICANCE: The development of a diatom shuttle-cloning vector and achieving diatom transformation will, for the first time, permit the techniques of modern molecular biology/genetics to be utilized with eukaryotic marine algae. Besides opening a door for biotechnological applications, molecular techniques will provide a sophisticated means of analyzing biochemical, physiological and/or biophysically relevant questions germane to algal problems associated with marine issues such as biofouling or remote sensing.

ANNUAL PROGRESS REPORT

GRANT #: R&T CODE: 441z014

PRINCIPAL INVESTIGATOR: Dr. Saul Roseman

INSTITUTION: The John Hopkins University

GRANT TITLE: Chitin Utilization by Marine Bacteria:  
Physiology and Molecular Mechanisms

REPORTING PERIOD: 15 April 1991 - 1 July 1991

AWARD PERIOD: 15 April 1991 - 14 March 1992

OBJECTIVE: To determine the regulation of genes that encode chitinase and to relate these modes of regulation to control of oligosaccharide uptake, to overall cellular carbon and energy metabolism, and to the linkages between these processes and positive chemotaxis of marine Vibrios to chitin-containing particles.

ACCOMPLISHMENTS: This is a new grant.

# ANNUAL PROGRESS REPORT

GRANT #: N00014-89-C-0085

R&T CODE: 4412060

PRINCIPAL INVESTIGATOR: Nahid S. Waleh

INSTITUTE: SRI International

GRANT TITLE: Genetic Engineering of Single-Domain Magnetic Particles

REPORTING PERIOD: 1 June 1990 - 15 May 1991

AWARD PERIOD: 1 March 1989 - 29 February 1992

OBJECTIVE: To identify and clone the genes of iron-uptake in *Aquaspirillum magnetotacticum* as a first step to understand the mechanism by which these bacteria synthesize single-domain magnetic particles.

ACCOMPLISHMENTS (last 12 months):

Cloning of a sequence of *Aquaspirillum magnetotacticum* that complements the *aroD* gene of *E. coli* and allows growth of *E. coli* *fep ent* in the presence of 2,2'-dipyridyl

A library prepared from the DNA of *A. magnetotacticum* in c2RB cosmid was screened by two different methods, A and B. In method A, the library was screened for the presence of sequences that would allow growth of *E. coli* strain HB101 *fepA entA* in the presence of inhibitory concentrations of 2, 2'-dipyridyl. This strain is iron-uptake deficient because it lacks the receptor (*fepA*) and one of the biosynthetic genes (*entA*) of enterochelin. In parallel (method B), the library was screened for sequences that would complement the *aroD* function of *E. coli* CL451 (=LE392 *aroD::Tn10*). The *aroD* gene product of *E. coli* codes for the enzyme 3-dehydroquinase which catalyzes the third step of the early common pathway for the biosynthesis of chorismic acid, precursor of aromatic amino acids and enterochelin. Enterochelin, a phenolate siderophore, is synthesized by *E. coli* and many gram-negative bacteria.

A 2kb DNA fragment was isolated from each set of screening experiments. Since fragment isolated by method B also allowed growth in the presence of inhibitory concentrations of 2,2'-dipyridyl, fragment of method A was tested for its ability to confer the Aro<sup>+</sup> phenotype. Upon transformation, CL451 transformants grew in the absence of aromatic amino acids and were iron-uptake proficient. Sequence data (over 1kb) indicated that the two fragments were identical.

The cloned 2kb fragment also complemented the *aroD* function of *Salmonella typhimurium*. The *S. typhimurium* *aroD* mutants transformed with the 2kb DNA fragment grew on minimal medium without aromatic amino acids and regained their ability to remove iron from the medium.

The cloned fragment did not show any DNA homology with the *aroD* gene of *E. coli* or the *qa2* gene of *Neurospora crassa* (as determined by Southern blot experiments). The *qa2* gene which codes for catabolic 3-dehydroquinase is a member of a gene cluster which codes for five central steps of the fungal shikimate pathway. The *qa2* gene complements *E. coli* *aroD* mutants that lack biosynthetic 3-dehydroquinase activity. The *aroD*- and *qa2*-coded enzymes are currently placed in separate classes of 3-dehydroquinases. A third class of dehydroquinases occurs in some plants where the enzyme is bifunctional. Comparison of the amino acid sequence of the *A. magnetotacticum* "*aroD-qa2*" gene product with other dehydroquinases is vital for the classification of this enzyme. Whether the *aroD* function and the chelating property of the cloned fragment are associated with one or two gene-products should await the results of the complete sequence data.



#### Isolation of a non-magnetotactic mutant strain of *A. magnetotacticum*

A mutant strain was isolated from an electroporated culture of *A. magnetotacticum* that was no longer magnetotactic. This mutant strain did not carry any plasmid and was sensitive to kanamycin, the resistance marker of the electroporating plasmid. The non-magnetic mutant strain was stable and did not revert to magnetic cells upon subculturing. Electron microscopic studies revealed that the non-magnetic mutant cells did not produce any magnetic particles. The protein profile of the mutant differed from that of the wild type strain. For example, a major cytoplasmic protein present in the magnetic cells was absent in the non-magnetic mutant strain. Further characterization of this mutant strain is currently in progress.

#### Purification of an iron-binding protein from the cytoplasmic fraction of *A. magnetotacticum*

We have initiated studies to characterize the cellular proteins of *A. magnetotacticum* that participate in iron transfer and synthesis of magnetic particles. Using immobilized metal-ion affinity chromatography, we have been able to identify several proteins from the total cell extract of *A. magnetotacticum* that show strong binding affinity for metal iron. One of these proteins, associated with the cytoplasmic fraction, has been purified to homogeneity by reverse phase HPLC. This protein which accounts for about 17% of the cytoplasmic protein fraction is absent in a nonmagnetic mutant strain. It has a molecular weight of about 16.5 KDa and pI of 6.2.

The sequence of the first 20 N-terminal amino acids of this protein has been determined. Two degenerate oligonucleotide fragments complementary to the N-terminal region have been constructed and used as probes in screening a lambda gt10 library for cloning its coding sequence.

#### Growth of *A. magnetotacticum* in the presence of oxyrase

*A. magnetotacticum* is a microaerophilic organism requiring 0.5-0.6% oxygen for growth and synthesis of magnetic particles. In our laboratory, this bacterium is routinely grown in nitrogen-gassed liquid medium prepared in a sealed bottle or in solid medium in a chamber with about 0.6% oxygen. Because these procedures are laborious and not suitable for large-scale experimentation, it was desirable to identify simpler methods for growing this organism.

Oxyrase (trade name) is an *E. coli* enzyme which in the presence of lactate or succinate removes oxygen from the medium. The effect of various concentrations of Oxyrase on growth of *A. magnetotacticum* on solid medium was investigated. Our results indicated that Oxyrase at concentrations between 0.06 to 0.12 units/ml promoted growth of *A. magnetotacticum*. Higher concentrations of Oxyrase had inhibitory effects. Under the microscope, *A. magnetotacticum* colonies had "snowflake" appearance with a dark brown center.

**SIGNIFICANCE:** The study of iron assimilation in magnetotactic bacteria will provide valuable insight into the mechanism by which these bacteria process large quantities of iron in order to synthesize single-domain magnetic particles.

**WORK PLAN (next 12 months):** Our work plan for next year includes: to clone and characterize the protein that we have identified from the cytoplasmic protein fraction of *A. magnetotacticum*; develop a genetic transfer system in *A. magnetotacticum*; isolate non-magnetic mutants of *A. magnetotacticum* by transposon mutagenesis; characterize the non-magnetic mutant strain that we have already isolated and described in this report.

**PUBLICATIONS AND REPORTS:** none

## ANNUAL PROGRESS REPORT

GRANT #: N00014-87-K-0012

R&T CODE: 4413022

PRINCIPAL INVESTIGATOR: David C. White, Marc W. Mittelman

INSTITUTION: Institute for Applied Microbiology, Univ. Tenn.

GRANT TITLE: Joint Program on Molecular Biology of Marine Organisms

REPORTING PERIOD: 1 July 1990 - 30 June 1991 (12 months)

AWARD PERIOD: 1 October 1986 - 30 September 1991

### OBJECTIVES: (of the UTK portion)

- 1) Develop non-destructive on-line and high resolution monitors for the formation, persistence and activity of microbial biofilms as they are affected by the shear gradients, substratum chemistry and heterogeneity, bulk phase chemistry, and genetic composition of the inoculum.
- 2) Utilize the techniques to define the molecular mechanisms involved in the adhesion of specific bacteria or consortia to substrata for insight into the mechanisms for modulating adhesion.
- 3) To genetically manipulate and analyze specific mutants of marine bacteria that have different properties relative to attraction and adhesion to substrata.
- 4) To train graduate and post-doctoral researchers in the application of techniques and instrumentation to important problems of marine research.

### ACCOMPLISHMENTS:

On-line, non-destructive monitoring devices based on changes in attached biomass (sensitive to  $10^4$  cells/cm<sup>2</sup>) with the quartz crystal microbalance (QCM), and the attenuated total reflectance Fourier transform infrared spectrometer (ATR-FTIR) have proven successful (6,8,12). An on-line adhesion assay has been developed based upon bioluminescence of a lux construct of Pseudomonas fluorescens associated with surfaces in flowing systems (14,20). Classification of methane-oxidizing bacteria with the high resolution signature biomarker technology based on phospholipid ester-linked fatty acids (PLFA) has been documented to correspond to 16S RNA sequence phylogenetic relationships (10). PLFA analysis has been semiautomated by semicritical fluid extraction (SFE)/derivatization. Studies using non-destructive electrochemical techniques applicable to microbially influenced corrosion (MIC) have shown the perturbation/response technique of electrochemical impedance spectroscopy has no detectable effects on biofilm composition and dynamics (13). Effects of biocides, photosynthetic microbes, specific combinations of microbes, passivation, on MIC have been reported (1, 3, 7, 9, 16). Spatial and temporal microbial activity associated with MIC has been mapped with the scanning vibrating electrode (11) and both MIC and non-destructive biofilm analysis have been reviewed (2, 15). A multi-electrode system for reproducibly measuring MIC has been reported (17). Archaeobacterial lipid biomarkers for biomass and differentiation based on SFC have been developed (5), validated by FTIR (8), and used to show biomass vs activity discontinuities in digesters (4, 18), and used to show environmental localization of ultrathermophiles in the hydrothermal vents (19).

### SIGNIFICANCE:

The systems developed have enabled quantitative analysis of biofilm formation. Specific bacteria added in sequence from continuous cultures in a laminar flow field reproducibly colonized replicate flow

cells; biomass and activity were affected by the order of addition. No spatial heterogeneity has yet been demonstrated based on substratum composition on a smooth surface. Inhomogeneities in microbial localization and activities on a smooth metal surface seem to promote MIC and different bacterial combinations (not necessarily the total biomass) show different degrees of MIC. The discontinuities between biomass and activity of organisms in biofilms for the eubacteria seem to be greatly magnified in the archaeobacteria. The ultrathermophiles actually exist in the metallic sulfides of the vent walls and show the same responses to temperature increases as they do in monoculture. One Ph.D. has been awarded and another will be within the next year from this work.

WORK PLAN (next 3 months):

The work will be finished up and the data correlated and published.

PUBLICATIONS AND REPORTS (9 of 22):

1. Franklin, M.J., D.E. Nivens, A.A. Vass, M.W. Mittelman, R.F. Jack, N.J.E. Dowling and D.C. White. 1991. Efficacy analyses of chlorine and chlorine/bromine treatments against bacteria associated with corroding steel. *Corrosion* 47: 128-134.
2. Geesey, G.G. and D.C. White. 1990. Determination of bacterial growth and activity at solid-liquid interfaces. *Ann. Rev. Microbial.* 44: 579-602.
3. Hedrick, D.B., A. Vass, B. Richards, W. Jewell, J.B. Guckert, and D.C. White. 1991. Starvation and overfeeding stress on microbial activities in high-solids high-yield methanogenic high yield digesters. *Biomass* 2: in press.
4. Hedrick, D.B., J.B. Guckert, and D.C. White. 1991. Archaeobacterial ether lipid diversity analyzed by supercritical fluid chromatography: Integration with a bacterial lipid protocol. *J. Lipid Res.* 32: 659-666.
5. Hedrick, D.B., D.E. Nivens, C. Stafford, and D.C. White. 1991. Rapid differentiation of Archaeobacteria from eubacteria by diffuse reflectance Fourier-transform infrared spectroscopic analysis of lipid preparations. *J. Microbiol. Methods* 13: 67-73.
6. Guckert, J.B., D.B. Ringelberg, D.C. White, R.S. Henson, B.J. Bratina. 1990. Membrane fatty acids as phenotypic approach to taxonomy of methylotrophs within the proteobacteria. *J. Gen. Microbiol.* 137: in press.
7. Franklin, M.J., D.E. Nivens, J.B. Guckert, and D.C. White. 1991. Effect of electrochemical impedance spectroscopy on microbial cell numbers, viability, and activity. *Corrosion* 49: in press.
8. Franklin, M.J., and D.C. White. 1991. Biocorrosion. *Current Opinior. in Biotechnology.* 2/3: June.
9. Mittelman, M.W., and D.C. White. 1990. The role of bacterial biofilms in contamination of process fluids by biological particulates. *In Particles in Gases and Liquids* (K.L. Mittal, ed.) Volume 2, Plenum Press, New York, pp. 33-50.

## **MOLECULAR RECOGNITION**

ARI (JOINT WITH CHEMISTRY DIVISION)

Scientific Officer: Dr. Harold J. Bright

PROGRAM OBJECTIVE: TO UNDERSTAND THE ATOMIC REQUISITES FOR SPECIFIC INTERMOLECULAR RECOGNITION, BINDING, AND REACTIVITY IN BIOMOLECULES.

NAVY OBJECTIVE: TO DEVELOP THE SCIENTIFIC BASIS FOR DESIGNING BIOSENSORS AND BIOCATALYSTS.

## ANNUAL PROGRESS REPORT

GRANT#: N00014-88-K-0181

R&T CODE: 441n007

PRINCIPAL INVESTIGATOR: H. Neal Bramson

INSTITUTION: University of Rochester

GRANT TITLE: A Modular Approach to Protein Design

REPORTING PERIOD: 7/1/90-6/13/91

AWARD PERIOD: 2/1/88-6/30/91

OBJECTIVE: To investigate protein-protein interactions. Peptides have each been designed to adopt particular conformations and to interact with a selected protein in a defined manner.

ACCOMPLISHMENTS (last 12 months): We have completed a study demonstrating that a fragment of the CD4 receptor, which is known from work in different laboratories to interact with the pp56<sup>lck</sup> tyrosine specific protein kinase, does in fact directly alter the reactivities of this enzyme. In the presence of a 30 residue synthetic peptide reproducing CD4 sequences the protein kinase has altered reactivities to peptide substrates. pp56<sup>lck</sup> catalyzes the phosphorylation of some sequences at least 20-fold more efficiently in the presence of this CD4 fragment as compared to in its absence, while the phosphorylation of other sequences are inhibited by more than 50%. This 50-fold switch implies that the kinase will catalyze the phosphorylation of different substrates when associated with the CD4 receptor than when free in the cell (which a significant fraction of the enzyme is). These changes in enzyme reactivity likely alter interactions with other proteins as well, and to investigate this possibility we are developing methodologies to detect such interactions. The approach that has proved most successful is to use peptides reproducing enzyme sequences large enough to adopt quasi-stable conformations, and to endow these peptides with a photoaffinity reagent which will modify any proteins that these peptides associate with. We have successfully employed such peptides to detect known protein-protein interactions, and are now ready to apply this technique to investigate pp56<sup>lck</sup> interactions with other proteins.

Our second project, which is to synthesize peptides that will interact with ribonuclease S to produce a chimeric protein that will bind to specific sequences of B DNA, has proved less successful. We have synthesized 4 peptides, of which 3 bind tightly (low micromolar binding constants) to Ribonuclease S. These peptides contain a portion of the enzyme active site, and must adopt at least a portion of the

desired conformation because they restore the bulk of enzyme activity. On the basis of CD spectroscopy, these 3 peptides do seem to form a portion of the desired helical structure, which means that we either get a portion of the structure (about 40%) all of the time or most of the structure a portion of the time. Unfortunately, none of these chimeric proteins can bind specific sequences of DNA, although they do bind nonspecifically. Dimerization of the peptide sequences, either directly or through peptide linkers had minimal effects. A likely reason for the failures to date are the rigid requirements of helix-turn-helix containing sequences for specific binding to DNA. These rigid requirements have been demonstrated by other laboratories over the last 18 months.

SIGNIFICANCE (of project 1): The finding that CD4 directly alters pp56<sup>lck</sup> reactivities is significant because the CD4 protein is a transmembrane receptor whose mechanism for signal transduction requires pp56<sup>lck</sup>. Our result implies that because the activities of this kinase seems to be constitutively altered upon binding to the receptor, CD4 likely effects signal transduction by localizing pp56<sup>lck</sup> in the vicinity of other proteins when CD4 and other proteins associate with the major histocompatibility complex of an antigen presenting cell. In addition, while pp56<sup>lck</sup> interacts with other receptors as well, the reactivity of this enzyme is likely optimized for different purposes through its interactions with each receptor type. Finally, our methods for identifying protein-protein interactions will likely be useful in a variety of studies.

WORK PLAN (future): We will continue to investigate CD4-pp56<sup>lck</sup> interactions and to identify protein-protein interactions that are important for signal transduction mechanisms.

PUBLICATIONS AND REPORTS (last 12 months):

1. Bramson, H. N., Casnellie, J. E., Nachod, H., Regan, L. M., and Sommers, C. Synthetic Fragments of the CD4 Receptor Cytoplasmic Domain and Large Polycations Alter the Activities of the pp56<sup>lck</sup> Tyrosine Protein Kinase. J. Biol. Chem., in press.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-C-0083

R&T CODE: 4412065

PRINCIPAL INVESTIGATOR: W. F. DeGrado

INSTITUTE: DuPont Merck Pharmaceutical Company, Biotechnology  
Department

GRANT TITLE: Bioorganic Models for Protein Ion Channels

REPORT PERIOD: 1 April 1990 - 30 March 1991

AWARD PERIOD: 1 April 1990 - 30 March 1992

OBJECTIVE: To investigate the structural features responsible for the conduction of ions through ion channel proteins; to design metal-binding peptides; and to develop novel biosensing devices.

### PROGRESS (year 1):

*Spectroscopic studies* We have prepared seven variants of the peptide,  $H_2N-(LeuSerSerLeuLeuSerLeu)_3-CONH_2$  in which a residue in the central heptad has been replaced by Trp. Fluorescence measurement indicate that the peptide binds to phospholipid (PL) vesicles with its helical axis parallel to the surface of the membrane. Quenching experiments with nitroxide-labeled phospholipids indicate that the voltage-dependence of channel opening in planar bilayers involves a voltage-induced change in peptide orientation from a surface to a transbilayer configuration.

*Modulating channel lifetime* We have increased the mean open time of our peptide channels ~200-fold by designing longer, helical peptides whose interaction surface extends beyond the hydrophobic side of the bilayer.

*Template-Assembled Channels* Toward our goal of designing channel-forming helix-bundles of defined size the peptide  $(LSSL SL)_3$ , and derivatives thereof, were reacted with a  $\beta$ -cyclodextrin. The tryptophan residue of the cyclodextrin served as a chromophore and thus facilitated the monitoring of the coupling reaction. The results are very encouraging. We used a tetra-phenylporphyrin derivative (provided by Dr. Groves et al., Princeton University) to make a tetrametric  $\alpha$ -helix assembly. The isolated bundle shows significant  $\alpha$ -helix content when inserted into vesicles and preliminary data indicate that it is capable of passing protons across phospholipid bilayers. The next goal will be to do specific amino acid substitutions in the peptide, attach it to the template, and evaluate the effects of the amino acid side chain on ion conductance and selectivity.

*Biosensing devices.* Two biotinylated ion channel peptides have been synthesized:

Biotin--Linker-- $(LeuSerSerLeuLeuSerLeu)_3-CONH_2$   
Biotin--Linker--Lys-Glu-Glu-Gly-Gly-Pro-Leu- $(LeuSerSerLeuLeuSerLeu)_3-CONH_2$

Both peptides form channels in planar bilayers. No major change in conductance properties were determined when avidin was added, even though it could be shown that avidin bound the biotin. In related work, a copper-binding peptide (His-Gly-Gly) was added in place of biotin to the peptides. Again, no major change in conductance was observed when  $Cu^{2+}$  was added. Therefore, we have abandoned work on this subject.

*De Novo Design of metal Binding Sites in Proteins* In previous work, we designed a  $Zn^{2+}$ -binding site in water-soluble four-helix bundle protein

by introducing two His residues on one helix and a third His on a linked helix. In this period we have studied the folding properties of the designed protein. In the absence of metals, the protein shows an NMR spectrum consistent with a "molten globule" structure. Upon addition of metal ions, the protein becomes considerably more ordered as assessed by a dramatic increase in the dispersion of its NMR spectrum. Finally, we have extended our original design to include a 2-His, 1-Glu  $Zn^{2+}$ -binding protein.

We have also begun to work on the design of a 2-His, 1-Cys copper-binding protein intended to mimic the properties of blue copper proteins. Physical and spectroscopic analysis will be carried out in the next year.

SIGNIFICANCE: The design of ion channel peptides should lead to a better understanding of the mechanism of natural ion channel proteins, and may also lead to the development of elements to be used in biosensing devices. In addition, our design of a metal-binding protein should help decipher the mechanisms of cation selectivity in natural systems, and may be elaborated into sensing devices.

WORK PLAN (next 12 months): The objectives of year 2 are: to better characterize the properties of our designed peptide channels, design template-assembled channels, design new metal-binding proteins, and characterize the conformational properties of metal-binding proteins.

PUBLICATIONS AND REPORTS (last 12 months):

Handel, T. and DeGrado, W.F. "De Novo Design of a  $Zn^{2+}$ -Binding Protein" *J. Amer. Chem. Soc.*, 112, 6710 (1990).

Osterhaut, J.J., Jr., Handel, T., Na, G., Toumadje, A., Long, R.C., Connolly, P.J., Hoch, J.C., Johnson, W.C., Jr., Live, D., DeGrado, W.F. "Characterization of a Peptide Designed to Form a Four-Helix Bundle" in press in *J. Amer. Chem. Soc.*



## ANNUAL PROGRESS REPORT

GRANT NUMBER: N00014-89-J-1174

R&T CODE 441n011

PRINCIPAL INVESTIGATOR: Elizabeth D. Getzoff

INSTITUTION: The Scripps Research Institute

GRANT TITLE: Defining Protein Electrostatic Recognition Processes

REPORTING PERIOD: 30 June 1990 - 1 June 1991 (11 months)

AWARD PERIOD: December 1988 - 30 November 1991

**OBJECTIVE:** The design and application of a tightly coupled computational and interactive computer graphics approach to elucidate the nature of electrostatic forces controlling the interaction of three general classes of electrostatic recognition processes: stable, catalytic and transient binding complexes.

### ACCOMPLISHMENTS (last 11 months):

Precollision Orientation in a Stable Binding Complex. The TURNIP program determines the most favorable precollision orientations for two molecules maintained at a specified distance by a systematic search of all orientations and evaluation of the resulting electrostatic interactions. TURNIP was applied to the investigation of formation of the stable complex between the HyHEL-5 antibody and its protein antigen lysozyme. Computer graphics analysis of the electrostatic fields suggested that the association of these two molecules might be substantially aided by electrostatic forces. The most prominent electrostatic feature of lysozyme was a large positive patch formed by three alpha helices roughly bounded by a triangle made up of the C $\alpha$  atoms of residues 3, 30, and 120. Interestingly, none of the three anti-lysozyme antibodies that have been crystallographically characterized in complexes with lysozyme (HyHEL10, D1.3, or HyHEL5) recognize this positive patch of the molecule. The Fv of the HyHEL5 antibody has as its major electrostatic feature a negative potential at the binding site. The electrostatic features of the antibody are much weaker than those seen for lysozyme. This is probably partly due to its smaller overall charge (-1) and to the large number of noncharged residues in the binding site, such as Tyr and Trp, which may function to form a large van der Waals surface with antigen. A TURNIP calculation performed with 6Å extended surfaces maintained a constant distance of 12Å between the two molecular surfaces and showed a disperse pattern of orientations. A second calculation was performed with 3Å extended surfaces, maintaining a distance of 6Å between the molecular surfaces. In the energetically most favorable orientations, the surface points of lysozyme that are part of the large positive patch were the closest to the antibody; however, Arg 45 and Arg 68 (residues of lysozyme in the interface of the HyHEL5 complex) were found at about a 90° angle from the intermolecular axis and were distributed radially around that axis. This indicates that electrostatic forces may help to orient the lysozyme towards the antibody binding site, but that a 90° rotation of the antigen relative to the antibody occurs during complex formation.

#### Importance of a Constant Separation Distance for Energy Evaluation.

Application of TURNIP to this complex showed that a mechanism for maintaining a constant minimum separation distance between the two asymmetric molecules was essential. Now, when interpenetration of the expanded surfaces occurs the moving molecule is moved out in steps of one half of the electrostatic grid spacing until interpenetration ceases. In the plastocyanin/cytochrome *c* system (Roberts et al., 1991) orientations requiring substantial move out distances could be as energetically favorable as those without. In the most energetically favored complexes calculated without the move out algorithm, lysozyme was centered over the edge of the HyHEL5 binding site. Thus, a constant minimum separation distance seems essential for the algorithm to work robustly.

Computer Program Development. We are continuing development of the graphics program Flex, for display of molecular dynamics and normal mode motions. Flex has been rewritten using the XView graphics library and the X-Windows protocol (developed at MIT), so that it runs on any machine with XView, for example Silicon Graphics, Stardent, and Sun SPARC graphics workstations. The X-Windows protocol allows rapid communications between machines such that Flex can be run on other machines, such as our Cray YMP or Convex C2 supercomputers, and viewed on a desktop workstation.

SIGNIFICANCE: Unlike the transient electron-transfer interactions previously investigated with TURNIP, in which electrostatic forces are known to enhance diffusion, the formation of this stable antibody-antigen complex appears to be only partially directed by electrostatic forces. Long-distance electrostatic forces may guide lysozyme toward the HyHEL5 binding site, but do not fine tune its orientation. This is consistent with the view that burying hydrophobic surfaces in the antibody-antigen interface is important for the stability of the final complex.

WORK PLAN: Next, the systematic search program TURNIP will be applied to formation of the tetramer of plasmid dihydrofolate reductase and its complex with NADPH. Also, the TURNIP program user interface will continue to be developed at the San Diego Supercomputer Center.

#### PUBLICATIONS AND REPORTS:

##### 1) Papers (copies enclosed):

Fisher, C. L., Tainer, J. A., Pique, M. E., and Getzoff, E. D. (1990) "Visualization of Molecular Flexibility and Its Effects on Electrostatic Recognition", *J. Mol. Graph.*, 8, 125.

Roberts, V. A., Freeman, H. C., Olson, A. J., Tainer, J. A., and Getzoff, E. D. (1991) "Electrostatic Orientation of the Electron-Transfer Complex Between Plastocyanin and Cytochrome *c*", *J. Biol. Chem.*, 266, in press.

##### 2) Abstracts (copies enclosed):

Roberts, V. A., Siani, M. A., Tainer, J. A., Freeman, H. C., and Getzoff, E. D. "Electrostatic Orientation of the Electron-Transfer Complex of Plastocyanin and Cytochrome *c*", Fourth Symposium of the Protein Society, San Diego, CA, August 11-15, 1990.

Pique, M. E., Macke, T. J., and Arvai, A. S. "Flex: A light weight molecular display program", Tenth International Meeting of the Molecular Graphics Society, Chapel Hill, NC, May 13-17, 1991.

## ANNUAL PROGRESS REPORT

GRANT # N00014-89-J-1167

R&T CODE 441 N012-01

PRINCIPAL INVESTIGATOR: Michael E. Hogan

INSTITUTION: Baylor College of Medicine

GRANT TITLE: The Design of Oligonucleotides Which Attack Specific Gene Targets

REPORTING PERIOD: May 1990-June 1991

AWARD PERIOD: December 1988 - November 1991

OBJECTIVE: To study the site selectivity of triplex forming oligonucleotides (TFOs) under conditions of physiological pH, employing a combination of molecular modeling, physical and biological experimentation. That understanding will be used to design TFOs with the capacity to bind to discrete gene targets.

### ACCOMPLISHMENTS (last 12 months):

#### **I. Demonstration of triplex formation in living cells.**

In the original application, we set out to determine if TFO binding to a promoter-enhancer region could be used as a mechanism to selectively inhibit gene expression in a living cell. As chronicled in our earlier progress reports, we believe that the preliminary stage of the work has been successful and that, in a manuscript which has been accepted for publication in PNAS this year (Gary Felsenfeld is the corresponding member), we have provided the first evidence for gene selective inhibition by triplex formation in a living cell.

More recently, the preliminary phase of a similar study was completed for the human interleukin 2 receptor (IL2R) gene and has been accepted for publication in Nucleic Acids Research. In that work, we have provided data to suggest that TFO binding to the promoter region of the IL2R gene can block its transcription in primary human T cells.

#### **II. Exploration of structure/affinity relationships for TFOs.**

In the original Science paper, we described a first generation TFO based upon GGC TAT and AAT triplet formation in an overall binding motif in which the third strand lays parallel with respect to the more purine rich strand of the underlying duplex. Subsequently, we have determined that a much preferred binding motif is one which includes only GGC and TAT triplets in a complex in which the third strand has a net antiparallel orientation relative to the purine rich strand of the duplex.

The existence of this new class of triple helix was described in previous ONR progress reports, our publication in the 23rd Jerusalem Symposium on The Molecular Basis of Specificity in Nucleic Acid-Drug Interactions and in a manuscript which is under review in Biochemistry.

During the past 12 month period of ONR support we have begun to explore the use of non-standard nucleoside substituents within the antiparallel motif which will allow

for stable TFO binding to DNA sequences which are not purine rich.

This program of chemical refinement is focused on three projects: design and use of novel G and T base homologues, the implementation of an altered sugar moiety and upon the design and implementation of internucleotide linkages with size, polarity and/or charge which is significantly different than the standard 5'-3' phosphodiester.

#### **SIGNIFICANCE:**

The significance of the past year of work is that it has shown that TFOs can now be considered to be agents which can be used to modulate gene expression in living cells.

#### **WORK PLAN:**

The work we propose for the final six months of ONR support falls into two categories:

**Synthesis of "Stretched" nucleoside homologues in triplex forming oligonucleotides.** We have developed a method to synthesize "stretched" nucleosides directly from guanosine and thymidine with high efficiency in a simple two-step procedure. The resulting nucleoside building blocks possess an 8-bond phosphodiester linkage. One goal of the program for ONR support will be to evaluate the utility of these stretched nucleoside modifications in TFOs, at sites where polypurine regions have been interrupted by T or C.

**Synthesis of Oligonucleotides with phosphate-free backbones.** To achieve the appropriate internucleotide span and to provide for strong hydration and other chemical characteristics of importance, we have begun to develop a new oligonucleoside polymer chemistry which employs an amino acid moiety as the internucleotide link.

Preliminary studies have been completed for the corresponding glycine-thymidine (T'gly) polymers which suggest that they can be synthesized in high purity and length-fractionated by HPLC methods. We have shown that they form duplexes with poly-dA which are nearly as stable as that formed with the corresponding unmodified oligo-dT.

#### **PUBLICATIONS AND REPORTS:**

E.J. Postel, S.J. Flint, D.J. Kessler & M.E. Hogan (1991) Evidence that a TFO Binds to the c-myc Promoter in HeLa Cells, Thereby Reducing c-myc mRNA Levels. PNAS in the press

F.M. Orson, D.W. Thomas, W.M. McShan, D.J. Kessler & M.E. Hogan (1991) Inhibition of IL-2 $\alpha$  mRNA transcription by promoter region collinear triplex formation in lymphocytes. Nucleic Acids Res. (in press)

## FINAL REPORT

GRANT #: N00014-88-K-0201

R&T CODE: 441n003

PRINCIPAL INVESTIGATOR: Paul B. Hopkins

INSTITUTION: University of Washington

GRANT TITLE: Control of Synthetic Peptide Tertiary...

AWARD PERIOD: 1 February 1988 - 31 January 1991

OBJECTIVE: The design, synthesis, and structural characterization of peptides of 10 to 35 residues with stable secondary and/or tertiary structure.

ACCOMPLISHMENTS (1 Feb 1988 - 31 Jan 1991): The annual reports should be referred to for further detail of the studies described in general terms below.

As originally proposed, it was our goal to induce helical structure into short peptides by introducing a covalent crosslink linking adjacent backbones in a putative  $\alpha$ -helical structure. Several such compounds were synthesized, and their conformational properties evaluated using circular dichroism. It was apparent from that work that rather than stabilizing helical structure, we had in fact destabilized helical structure. This was particularly discouraging, in that the syntheses of the required compounds had been quite elaborate and had required considerable time. The concept appeared fundamentally sound, but execution was proving technically difficult. For this reason, we changed our approach to one in which a pair of metal binding residues was placed in short peptides, with two or three residues intervening. It was our hope that on addition of metal ions, these two ligands would simultaneously bind to a single metal ion, resulting in enhanced  $\alpha$ -helicity. Several advantages would accrue from this approach. Firstly, it is substantially simpler to synthesize acyclic peptides. Furthermore, because it is easy to vary the length of the tether which holds the ligands to the backbone, and a variety of metals which favor different coordination geometries exist, there would be available a variety of combinations to allow us to explore a considerable number of structures. This approach proved successful. A publication appeared in the *Journal of the American Chemical Society* late in 1990 that reported the synthesis of eight peptides containing a pair of metal binding amino acids, along with two control peptides. Conformational studies of these peptides by circular dichroism clearly indicated that some combinations of peptide with metal ions resulted in distinct and significant enhancement of helicity. The most astonishing example of this was an 11-residue peptide which in the absence of metal

ions adopted a random coil conformation but in the presence of metal ions was at least 80% helical at 4 °C.

There are two directions of obvious interest given these findings. The first is to understand the physical origin of this helix stabilization. To explore this, we have determined the enthalpy and entropy changes associated with apo- and metal-bound peptides in the course of their coil to helix transition. Several such thermodynamic quantities have been measured and in general terms, we find that the metal-bound peptides actually form helices with somewhat less favorable enthalpy, consistent with the crosslink being disruptive of helical structure. Offsetting this, however, is a diminution in the extent to which helix formation is entropically unfavorable. The latter effect dominates in those peptides we have studied and thus their metal-bound forms are of higher helix content than the corresponding apo-peptides. We have measured the binding constant for one of these peptides to cadmium, and find it to have a value of  $10^9$ . We've also investigated, by means of theory, one further unexpected finding. Whereas a 23 residue peptide containing a particular set of metal binding residues changed its helix content from 50% to roughly 80% on addition of cadmium, a truncated version of this peptide which was only 11 residues in length, changed its helicity from a negligible quantity to almost 75%. Why is the shorter peptide more strongly changed in conformation than the longer peptide? We have modified the zipper model of J.A. Schellman to allow for the incorporation of a single residue of very high nucleation capability. We find that this model does in fact predict the conformational changes associated with nucleation will be greatest in short peptides.

The second direction which we recognize is of interest has not yet been pursued but will be pursued in the near future. This involves use of these stabilized helices as recognition elements in biological systems. For example, it is easy to imagine use of these stabled helices as recognition elements in the delivery of agents to double helical DNA.

**SIGNIFICANCE:** The helices described herein are the smallest stable helices currently known. They may posses utility in study of helical structure in solution or in crystals. As noted above they may prove useful as structural modules in the synthesis of larger molecules which have function. With the exception of recent reports out of the Scripps Laboratory from Ghadiri and Choi, all other examples of stabilized helices reported to date rely upon aggregation of more than one helix (three or four) to stabilize helical structure. As such, the helices reported herein might be called "naked" helices, simpler in structure and simpler to

study. An advantage of our helices over those reported by the Scripps group is their high metal binding constants which allow, for all practical purposes, a single equivalent of metal ion to be added to drive essentially complete formation of helices.

PUBLICATIONS AND REPORTS:

Hopkins, P.B., Ruan, F., Chen, Y., Sasaki, T. and Itoh, K., "Metal Ion-Induced  $\alpha$ -Helicity in Synthetic Peptides Containing Unnatural, Metal-Ligating Residues," Abstract, 200th National Meeting of the American Chemical Society, Washington, D.C., August 26-31, 1990.

Hopkins, P.B., Ruan, F. and Chen, Y., "Metal Ion-Induced  $\alpha$ -Helicity in Synthetic Peptides Containing Unnatural, Metal-Ligating Residues," *J.M. Chem. Soc.*, 1990, **112**, 9403.

Ruan, F., Chen, Y., Itoh, K., Sasaki, T. and Hopkins, P., "Synthesis of Peptides Containing Unnatural, Metal-Ligating Residues: Aminodiacetic Acid as a Peptide Side Chain," submitted to *J. Org. Chem.*

Chen, Y., Ruan, F. and Hopkins, P.B., "Thermochemistry of Metal Ion Stabilized Peptide Helices," in preparation for submission to *J. Am. Chem. Soc.*

ANNUAL PROGRESS REPORT

Grant No.: N00014-89-J-1434

Principle Investigator: Peter T. Lansbury, Jr.

Institution: Massachusetts Institute of Technology

Grant Title: Models of Glycoprotein Folding

Reporting Period: 1 June 1990 - 30 May 1991

Award Period: 1 Nov. 1988- 1 Nov. 1991

Objective: To elucidate the basic principles of polysaccharide-protein interactions through the study of glycopeptide models.

Accomplishments (last 12 months): We have developed a practical method for the synthesis of glycopeptides. A portion of this work was published last year (*J. Org. Chem.*, enclosed). This chemistry gives us access to compounds which were previously extremely difficult to synthesize. At this time, two glycopeptides have been thoroughly analyzed by 2D  $^1\text{H}$ NMR. These molecules assume a turn-like structure in DMSO, as indicated by the detection of several NOE's. The complete elucidation of structure requires additional experiments. We have also completed the synthesis of a designed peptide which has been successfully glycosylated in a preliminary experiment.

In order to define the conformational requirements for glycosylation, we have synthesized two peptides with sequences derived from the glycoprotein ovalbumin. Both of these sequences contain a consensus glycosylation site, although one of these sites is not modified *in vivo*. Conformational studies are in progress to determine if this fact can be attributed to a structural difference between the two sequences.

Significance: We are currently able to synthesize a wide variety of glycopeptides for structural studies. These molecules are extremely difficult to make using previously existing methods.

Work Plan: A series of glycopeptides with natural and unnatural sequences are being synthesized. Systematic conformational studies (2D NMR, CD, FTIR) will elucidate the principles which govern protein-polysaccharide interactions. Studies of the ovalbumin peptides will focus on FTIR and CD spectroscopy. Our initial goal is to define environmental conditions which effect the two peptides differently. These conditions may be analogous to the conditions of *in vivo* glycosylation and glycoprotein folding.

Publications: "A Convergent Approach to the Chemical Synthesis of Asparagine-Linked Glycopeptides." Shimon T. Anisfeld and Peter T. Lansbury, Jr. *J. Org. Chem.* 1990, **55**, 5560.



## ANNUAL PROGRESS REPORT

GRANT # N00014-90-J-1407

R&T Code:441-3017---05

PRINCIPAL INVESTIGATOR: Harden M. McConnell

INSTITUTE: Stanford University

GRANT TITLE: Determination of the Structural Basis of Antibody Diversity Using NMR

PERIOD OF PERFORMANCE: 1 July 1990 - 30 June 1991

OBJECTIVE: To use NMR to determine the composition, structure, and kinetics of antibody combining sites

ACCOMPLISHMENTS: (since July 1990)

The principle overall accomplishment in this work has been the development of a practical recipe for obtaining unique kinetic and structural information on antibody combining sites in solution. This recipe has been applied to the antibody molecule AN02, but is doubtless quite general and applicable to many different antibodies. A most important finding has been made since our last report (1990 December 14). The proton resonance spectrum of a unique tyrosine residue in the combining site region has been particularly difficult to identify, and has finally been identified. This tyrosine is unique (among the half-dozen tyrosine residues in the combining site region) because it undergoes large chemical shifts on hapten binding, and strong line broadening on increasing the temperature. This tyrosine has been identified by single site mutagenesis, Tyr 31L → Phe 31L. This mutation results in the loss of the unique tyrosine resonance ("signal G") from the resonance spectrum. These results are displayed in Figure 1. In Figure 1 spectra A and A' give tyrosine proton signals in the empty combining site region, where A' is the spectrum of the mutant Tyr 31L → Phe 31L. Note that signal G has disappeared, and the other proton resonance signals undergo little change. Spectra B and B' give tyrosine signals for a binding site occupied by hapten. Again in the Tyr 31L → Phe 31L mutant, signal G has disappeared.

PUBLICATIONS AND REPORTS (last 12 months)

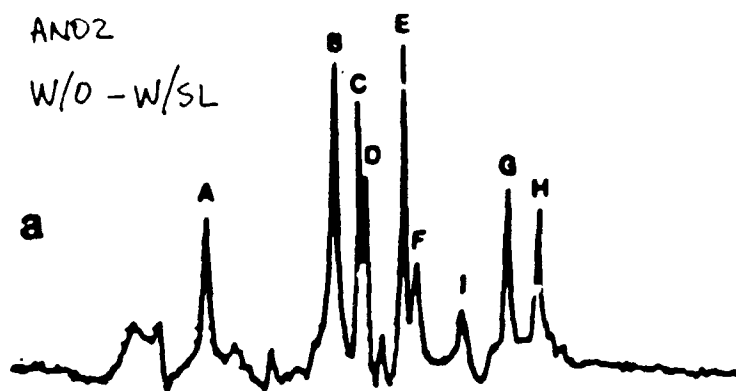
"Structural and kinetic studies of the Fab fragment of a monoclonal anti-spin label antibody by NMR", T.P. Theriault, G.S. Rule, D.J. Leahy, M. Levitt and H.M. McConnell. J. Mol. Biol. (in press).

AND2

W/O - W/SL

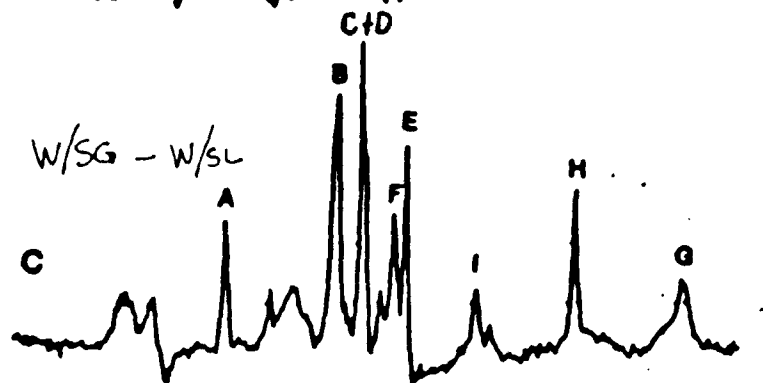
3.5

a



W/SG - W/SL

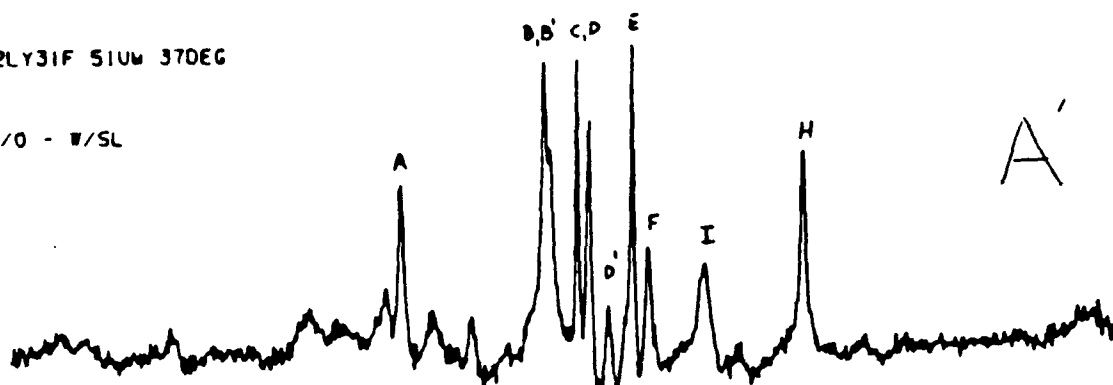
C



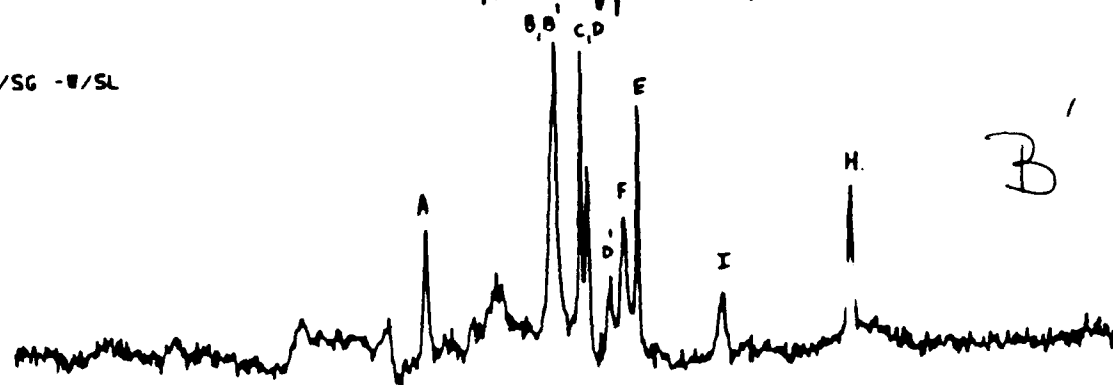
7.5 7.0 6.5 6.0

2LY31F 510W 37DEG

W/O - W/SL



W/SG - W/SL



7.6 7.2 6.8 6.4 6.0  
PPM

FIGURE 1

# ANNUAL PROGRESS REPORT

GRANT #: N00014-89-K-3025

R&T CODE: 441g012

PRINCIPAL INVESTIGATOR: Peter G. Schultz

INSTITUTE: University of California, Berkeley

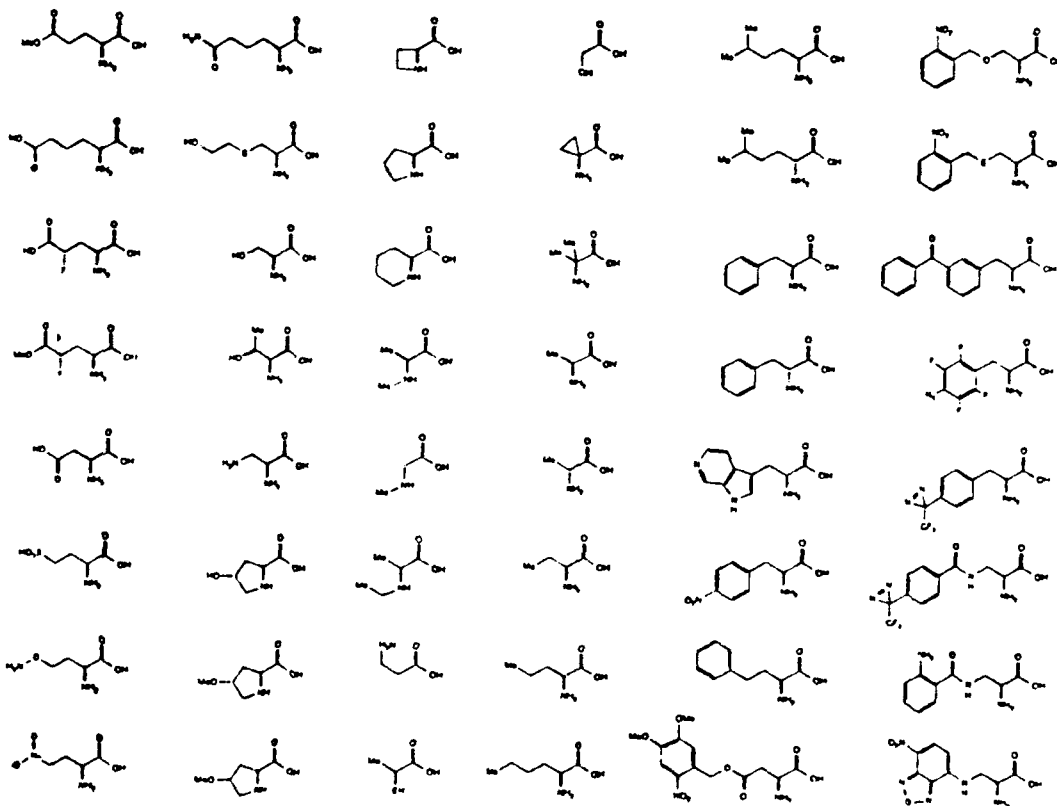
GRANT TITLE: Site Directed Mutagenesis With Unnatural Amino Acids

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 August 1989 - 31 July 1992

**OBJECTIVE:** The methodology for site-specific mutagenesis with unnatural amino acids will be optimized with regard to (a) suppressor tRNA generation (via runoff transcription and gene synthesis and expression), (b) tRNA aminoacylation (including exploration of acid, photochemical and redox labile protecting groups and enzymatic aminoacylation) and (c) *in vitro* protein synthesis (including optimization of transcriptional and translational signals, and alternative *in vitro* systems). We are synthesizing the requisite unnatural amino acids and generating mutants of 434 repressor, T4 lysozyme, sperm whale myoglobin, *ras* oncogene protein and ribonucleotide reductase in order to probe structure-function relationships in these enzymes.

**ACCOMPLISHMENTS (last 12 months):** In the last twelve months, we have continued to make improvements in the unnatural amino acids mutagenesis methodology. The following amino acids have successfully been loaded onto tRNAs (primarily via the use of photochemically removable protecting groups).



Large quantities (30mg) of suppressor tRNA can be easily and rapidly generated. In addition suppression efficiencies now average ~50%. We are now capable (in theory) of generating 0.5 mg quantities of protein. We have also generated a series of mutations in ras oncogene protein and T4 lysozyme. The T4 lysozyme mutations include the following: Alanine → lactic acid (ester backbone), pipecolic acid (6-membered ring proline analogue), cyclopropyl glycine, cyclohexylglycine, aminobutyric acid (disubstituted amino acids), N-methylalanine (secondary amides); Glutamate 11 → homoglutamate, (isoelectronic homologue), flouroglutamate (altered  $pK_A$ ), glutamate methylester; Asp 20 → nitrobenzylaspartate (photoactivatable caged protein). The catalytic properties and thermal stabilities of these proteins are currently being characterized.

Ras mutations included the following: Glutamine 61 → homoglutamine (homologue), Glycine 60 → glycolate ( $NH \rightarrow O$ ), N-methylglycine (secondary amide); Ala 59 → hydroxyethylcysteine (serine homologue); Glycine 12 → 3 and 4 substituted prolines, lactic acid, N-methylglycine. The effects of these substitutions (side chain and backbone) on the ability of ras to act as a signal switch are currently being evaluated.

WORK PLAN (next 12 months): We will characterize the catalytic, folding, and thermal stabilities of the ras and T4 mutants we have generated. In addition we will generate additional mutants of these proteins to study (1) the effects of backbone conformation on protein structure at turns and  $\alpha$ -helices, (2) the effects of "hole-filling" on protein stability, (3) protein-protein contacts in signal transduction cascades (using selectively incorporated photoaffinity labels) and (4) protein conformation changes (using fluorescent amino acids). We are also beginning to apply this methodology to the DNA binding protein 434 repressor (probe the nature of DNA protein recognition).

PUBLICATIONS AND REPORTS (last 12 months):

1. Mendel., Ellman, J., and Schultz, P.G. (1991) "Construction of a 'Caged Protein' by Site Directed Unnatural Amino Acid Mutagenesis", *J. Am. Chem. Soc.*, 11, 2758.
2. Ellman, J., Mendel, D., Anthony-Cahill, S., Noren, C.J., and Schultz, P.G. "A Biosynthetic Method for Introducing Unnatural Amino Acids Site Specifically Into Proteins", *Methods in Enzymology*, (in press).
3. Robertson, S.A., Ellman, J., and Schultz, P.G. (1991) "A General and Efficient Route for Chemical Aminoacylation of Transfer RNAs", *J. Am. Chem. Soc.*, 113, 2722.

# ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-3045

R&T CODE: 441g013

PRINCIPAL INVESTIGATOR: David S. Sigman

INSTITUTION: University of California, Los Angeles

GRANT TITLE: Design of Sequence-Specific Nucleases By Chemical and Genetic Modification of the *E. Coli Trp* Repressor

REPORTING PERIOD: 7/1/90 - 6/30/91

AWARD PERIOD: 8/1/89 - 7/31/92

## OBJECTIVE:

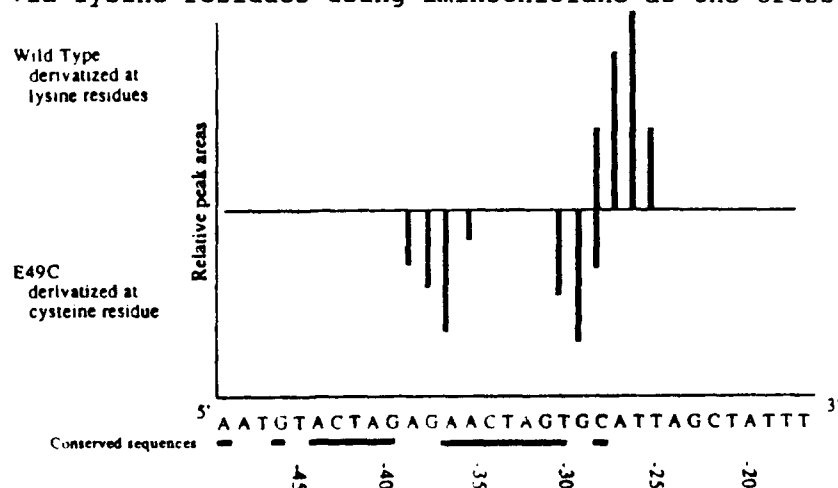
Preparation of a site-specific DNA scission reagent as a chemically homogeneous conjugate of the *E. coli trp* repressor with the chemical nuclease 1,10-phenanthroline-copper.

## ACCOMPLISHMENTS (last 12 months):

Examination of available *E. coli trp* repressor crystal structures indicate that our objective could be achieved by inserting a cysteine residue at either position 46 or 49. Alkylation of either of these unique cysteine residues with 5-iodoacetamido-1,10-phenanthroline should provide chemically discrete scission reagents which bind DNA with high affinity and direct the nucleolytic activity to the oxidatively sensitive minor groove.

The mutagenesis at position 49 has been achieved and the protein (E49C) expressed in good yield from the same expression vector as the wild-type protein. Following purification of the mutant protein, its site-specific binding has been confirmed by gel retardation using the *aro H* operator as a probe. 5-Iodoacetamido-1,10-phenanthroline was then used to alkylate cys-49 and the specific binding of the chemically modified mutant protein was again confirmed by the gel retardation. Scission was activated by diffusion of the coreactants ( $\text{Cu}^{++}$ , thiol,  $\text{H}_2\text{O}_2$ ) into the gel matrix.

Two regions of scission are clearly observed within the DNase I footprinted region of the *aro H* operator sequence. This site directed scission is dependent upon the presence of both derivatized cysteine containing *trp* repressor and thiol. No scission is seen with mock derivatized wild type *trp* repressor, underivatized E49C or any co-purifying *E. coli* cell components. As indicated below, the scission pattern of E49C OP is distinct from that observed in our previous work when OP was linked via lysine residues using iminothiolane as the cross-linking agent.



#### SIGNIFICANCE:

The preliminary data clearly indicate that the *E. coli trp* repressor can be converted into a highly efficient nuclease activity using site-directed mutagenesis and chemical modification. The modified protein is chemically discrete and retains high affinity binding. Mutants of the protein will now be sought with different DNA binding specificities.

#### WORK PLAN (next 12 months):

Our goals for the next year include: a) further characterization of the reaction of 1,10-phenanthroline derivatized E49C *E. coli trp* repressor with special reference to maximizing the scission by examining buffer composition, protein concentration, and reaction times; b) generation of the second mutant (D46C) and characterization of its reactivity; and c) developments of the methods for screening or selecting variants of these genetically engineered *E. coli trp* repressors with different DNA binding specificities.

#### PUBLICATIONS AND REPORTS (last 12 months):

1. Yoon, C., Kuwabara, M.D., Spassky, A., and Sigman, D.S. (1990) "Sequence Specificity of the Deoxyribonuclease Activity of 1,10-Phenanthroline-Copper Ion". *Biochem.* **29**, 2116-2121.
2. Bruice, T.W., Wise, J., and Sigman, D.S. (1990) "Engineering of a Semisynthetic Endonuclease Based on the Lambda Phage Cro Protein and Nuclease Activity of 1,10-Phenanthroline-Copper". *Biochem.* **29**, 2185-2186.
3. Thederahn, T., Spassky, A., Kuwabara, M.D., and Sigman, D.S. (1990) "Chemical Nuclease Activity of 5-Phenyl-1,10-Phenanthroline-Copper Ion Detects Intermediates in Transcription Initiation by *E. coli* RNA Polymerase". *Biochem. Biophys. Res. Commun.* **168**, 756-762.
4. Sigman, D.S. and Chen, C.-H. B. (1990) "Chemical Nucleases: New Reagents for Molecular Biology". *Ann. Rev. of Biochem.* **59**, 207-236.
5. Sigman, D.S. (1990) "Chemical Nucleases". *Biochem.* **29**, 9097-9105.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-88-0179

R & T CODE: 441n004

PRINCIPAL INVESTIGATOR: Douglas H. Turner

INSTITUTE: University of Rochester

GRANT TITLE: Molecular Basis of RNA Catalysis

PERIOD OF PERFORMANCE: 1 July 1990 - 28 February 1991

OBJECTIVE: To understand the mechanism and substrate binding for self-splicing reactions of intervening sequences (IVS) in RNA.

### ACCOMPLISHMENTS (6/30/90-2/28/91):

Dissociation constants at 15 °C were measured by equilibrium dialysis for the binding of rCrUrCrU, dCrUrCrU, rCdUrCrU, rCrUdCrU, and rCrUrCdU to the L-21 Sca I form of the self-splicing group I LSU intron from *Tetrahymena thermophila*. Substitution of deoxyribose for ribose in each of the middle two positions makes the free energy change for binding 1-2 kcal/mol less favorable, compared to about 0.3 kcal/mol less favorable for each of the terminal positions. Dissociation constants for binding of the same oligomers to rGGAGAA, a mimic of the ribozyme binding site, were measured by optical melting methods. Substitution of a single deoxyribose for ribose makes the free energy change for binding less favorable by 0.4-0.9 kcal/mol for this simple duplex formation. Comparison of the effects for binding to ribozyme and to rGGAGAA indicate that ribozyme specific tertiary interactions dependent on the middle two 2' OH groups of rCrUrCrU add about 2 kcal/mol of favorable free energy for binding to L-21 Sca I.

The dissociation constant measured for rCrUrCrU by equilibrium dialysis is about 200 times smaller than the  $K_m$  measured for the reaction of rCrUrCrU with UCGA catalyzed by L-21 Sca I. This raises the possibility that the tightest binding site is not the reactive site. In addition, rates of reaction under single turnover conditions at 50 mM  $Mg^{2+}$  are faster when CUCU is added before UCGA rather than vice versa. At 5 mM  $Mg^{2+}$ , rates are independent of the order of addition for CUCU and UCGA. This suggests a conformational change is required for reaction at 50 mM  $Mg^{2+}$  when UCGA is added first.

The secondary structure of L-21 Sca I was mapped at different  $Mg^{2+}$  concentrations and different temperatures. The results suggest the  $Mg^{2+}$  and temperature induced unfolding of L-21 Sca I is complicated.

## SIGNIFICANCE

This is the first equilibrium measurement that shows that 2'OH groups can contribute about 1 kcal/mol each to RNA substrate binding to a ribozyme. This confirms a suggestion we made earlier based on kinetic data (N. Sugimoto, M. Tomka, R. Kierzek, P. C. Bevilacqua, & D. H. Turner, *Nucleic Acids Res.* 17, 355-371 (1989)). The magnitude of the free energy contribution is similar to the free energy contribution deduced for a hydrogen bond in a base pair or GA mismatch (D. H. Turner, N. Sugimoto, R. Kierzek, & S. D. Dreiker, *J. Am. Chem. Soc.* 109, 3783-3785 (1987); J. SantaLucia, Jr., R. Kierzek, & D. H. Turner, *J. Am. Chem. Soc.* 113, 4313-4322 (1991)).

WORK PLAN: Not applicable. This contract is terminated.



**WATER AT BIOLOGICAL INTERFACES  
(NEW PROGRAM)**

**CORE PROGRAM**

**SCIENTIFIC OFFICER: DR. HAROLD J. BRIGHT**

**PROGRAM OBJECTIVE: TO DETERMINE THE NATURE AND FUNCTION OF WATER MOLECULES AT THE PROTEIN-SOLVENT INTERFACE.**

**NAVY OBJECTIVE: TO IMPROVE UNDERSTANDING OF SOLVENT-MEDIATED NON-COVALENT FORCES IN THE INTERACTION OF SENSORS AND BIOCATALYSTS WITH LIGANDS OF NAVAL INTEREST.**

**IMMUNOPHYSIOLOGY**

**CORE PROGRAM**

**SCIENTIFIC OFFICER: Dr. Jeannine Majde**

**BEGAN: OCTOBER 1, 1989**

**PROGRAM OBJECTIVE: TO INVESTIGATE THE INTERACTIONS OF THE IMMUNE SYSTEM AND THE NEUROENDOCRINE SYSTEM DURING THE ACUTE PHASE OF PHYSICAL TRAUMA, INFECTION OR WOUND REPAIR.**

**NAVY OBJECTIVE: TO PROVIDE NEW TARGETS AND STRATEGIES FOR TREATMENT OF SHOCK, TRAUMA, TISSUE INJURY AND ACUTE VIRAL INFECTION.**

ANNUAL PROGRESS REPORT

**Grant #:** N00014-91-J-1123

**Principal Investigator:** Dr. Carol A. Colton

**Institution:** Georgetown University Medical School

**Grant Title:** The role of interferon in the cellular response of the CNS macrophage, the microglia during injury and inflammation

**Reporting Period:** 1 January 1991 to 31 May 1991.

**Award Period:** Dec 1, 1991 to Nov 30, 1994

**Objective:** To investigate the action of interferon in the response of the brain to injury.

**Accomplishments (last 6 months):** We have examined the action of alpha/beta and gamma interferon (IFN) on the function of the CNS macrophage, the microglia. Two indices of microglial function have been studied, superoxide anion production and interleukin-1 production. The experiments were carried out on cultured microglia obtained from neonatal rat cerebral cortices. Although data from these experiments are currently being analyzed, several trends have appeared. In terms of superoxide anion production, treatment with alpha/beta IFN for 24, 48 or 72 hours increased phorbol myristate acetate (PMA) induced-superoxide production compared to PMA alone. A similar phenomenon is seen when gamma interferon is used to activate the microglia, but gamma IFN is effective at 10 times lower concentrations than alpha/beta IFN. A slightly different picture is seen when opsonized zymosan (OPZ) is used to stimulate superoxide anion production. In this case, only alpha/beta IFN treatment enhanced superoxide production compared to OPZ alone where a significant increase was seen at 48 hours only. Gamma IFN did not significantly potentiate OPZ-induced superoxide anion production at any concentration or time point studied.

We also examined the effect of alpha/beta IFN and gamma IFN on production of interleukin-1 (IL-1). IL-1 was measured in the supernatants of microglia treated with IFN or untreated controls using a sensitive bioassay (the D10 cell proliferation assay). Initial experiments indicated that IL-1 activity increased during stimulation with alpha/beta IFN. Since these experiments were performed using 10% fetal calf serum, they were redone using a serum-free media. In addition, all media and reagents were assayed for endogenous endotoxin using the Limulus Amoebocyte assay. Preliminary results from these studies indicate that alpha/beta IFN has no independent effect on microglial IL-1 production. The presence of a finite level of endotoxin (e.g. LPS) in the Sigma murine alpha/beta IFN may explain why a response to IFN was seen. LPS is a potent

stimulus for IL-1 production and frequently contaminates reagents or media. Gamma IFN had no effect on IL-1 production in either serum containing or serum free media.

**Significance:** The results to date indicate that both alpha/beta and gamma IFN potentiate superoxide anion release from microglia while neither may affect IL-1 production. In addition, it is clear that alpha/beta and gamma IFN have different modes of action.

**Work Plan:** (next 12 months) Objective #2 will be continued by examining the effects of IFN on protease production and phagocytosis. We are currently developing the zymography technique and will apply this to the study. Part of the difficulty of this technique is the amount of cells needed to get an adequate signal. Preliminary experiments indicate that more cells will be required than originally planned. In addition, we will finish the superoxide and IL-1 section of this objective by completing the time study.

**Publications and Abstracts:**

Colton, C., Keri, J., Gilbert, D. and Yao, J., The action of alpha/beta and gamma IFN on the CNS macrophage, the microglia, in preparation.

Keri, J., Chen, W., Monsky, W., Yao, J., Colton, C and Gilbert, D., Degradation of immobilized fibronectin-gelatin substrates by LPS and IL-1 stimulated microglia, Neurosci. Abst., Nov. 1991.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1311

R&T CODE: 4414902

PRINCIPAL INVESTIGATOR: Carl W. Dieffenbach, Ph.D.

INSTITUTION: Uniformed Services University of the Health Sciences and The Henry M. Jackson Foundation for the Advancement of Military Medicine

GRANT TITLE: Molecular Studies of Cytokine Induction

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD:

OBJECTIVE: To define the cytokine response of murine monocytes to challenge with several cytokine inducers. To investigate the role of specific double stranded RNA structures in this cytokine response.

ACCOMPLISHMENTS: We are currently investigating the cytokine response in two different cell systems. To date we have completed the comparison of lipopolysaccharide (LPS) and polyI:polyC as cytokine inducers in the murine monocytic cell line RAW. We have found that there are significant differences in which cytokines and the quantity of the cytokines that are induced. RNAPCR analysis (Figure 1) indicates that polyI:polyC stimulates the accumulation of IFNs  $\alpha$  and  $\beta$ , IL-1 $\alpha$  and a very low level of IL-6. LPS stimulates the accumulation of high levels of IFN $\beta$ , IL-1 $\alpha$ , IL-6. There was no induction of IFN $\alpha$  by LPS treatment.

Using this RNAPCR system we have initiated investigation of the cytokine induction in vitro and in vivo by influenza A virus. RNA prepared from mouse lungs of influenza A PR/8/34 infected and sham treated animals at 24 and 48 hrs post infection was used as starting material for the RNAPCR. The RNAPCR detected IFN $\alpha$ , TNF $\alpha$ , and IL-6 at 24 and 48 hrs of infection. IL-1 $\alpha$  was detectible only at 24hrs post infection. The control, glyceraldehyde-3-phosphate dehydrogenase was amplified in all three RNA samples. Additional studies on the mechanism of influenza induction will focus on the double stranded RNA (dsRNA) structures produced during virus infection.

We have also investigated the regulation of IFN gene expression in resident peritoneal macrophages (PM) of lps<sup>d</sup> and lps<sup>n</sup> mice. We have shown that in vitro treatment with LPS or IFN gamma results in increased accumulation of mRNA and expression of IFN $\beta$  in lps<sup>n</sup>, but not lps<sup>d</sup> PM. Nuclear run-on experiments showed no increased IFN $\beta$  transcription in the LPS treated cells. To further demonstrate that IFN $\beta$  production resulted from an increase in mRNA stability, we demonstrated that cycloheximide could also induce IFN $\beta$  mRNA accumulation. Interestingly, cycloheximide also induced IFN $\alpha$ -4 mRNA accumulation in the PM of both strains. Preliminary studies examined the stability of IFN $\beta$  mRNA in lps<sup>n</sup> and lps<sup>d</sup> PM. The IFN $\beta$  mRNA was rapidly turned over in the lps<sup>d</sup> PM but not the lps<sup>n</sup> peritoneal macrophages. These results indicate that a lack of IFN response in the lps<sup>d</sup> PM arises from an alteration in the machinery controlling the IFN $\beta$  mRNA, but not the IFN $\alpha$  mRNA. It is also of interest

to note that the IFN expression pattern of the RAW cells parallels the lps<sup>n</sup> PM.

SIGNIFICANCE: The expression of cytokines as analyzed in these systems will provide an understanding of the mechanisms controlling the synthesis and production of these important molecules. Through the characterization of the inducer and the type, pattern and quantity of cytokine made, general pathways of cytokine induction in macrophages, analogous to the Th-1 and Th-2 sets, can be derived. Additionally, this study will shed light of the role of these cytokines in disease processes.

WORK PLAN: The specific objectives for the next year are to examine in more detail the cytokine production in the RAW cells and determine the mechanisms (transcriptional or post-transcriptional) of cytokine induction. This study will be extended to include an investigation of the effects of cytokine pretreatment on the induction profile of LPS and polyI:C. The examination of mechanisms of control will also be determined in the PM, we will determine if the mechanisms operating in both the RAW and PM cells are similar.

Within the next year we will define at the molecular level the basis for the increased IFN $\beta$  mRNA stability in the lps<sup>n</sup> PM. We are currently focusing on RNA binding proteins that improve the stability of the IFN $\beta$  mRNA in vitro. This protein must be differentially expressed in lps<sup>n</sup> versus lps<sup>d</sup> PM.

As part of our ongoing study of dsRNA, we will develop an in vitro assay to examine the structure/function relationship of dsRNA and cytokine induction. This system will be based on the ability of specific RNA preparations to trigger the in vitro phosphorylation of the dsRNA-dependent protein kinase. We have obtained the full length kinase clone from Bryan R.G. Williams plan to express this clone in its inactivated state. It will become activated only through incubation with dsRNA.

We will also compare the of cytokine profiles induced by influenza virus that is not adapted with mouse adapted pathogenic forms. These studies will be performed in the mouse lung infection model. This will help define the role of the cytokine cascade in the pathogenesis of influenza. The quantity of specific dsRNA produced during the virus infection will be determined using the new in vitro assay. Differences in amounts of dsRNA produced may prove to be quite predictive of pathogenesis.

#### PUBLICATIONS AND ABSTRACTS

1. Gessani, S., Di Marzio, P., Dieffenbach, C.W., Baglioni, C., Belardelli, F. (1990) Posttranscriptional regulation of IFN- $\beta$  mRNA in murine macrophages. J. IFN. Res. 10: S40.
2. Bickel, M., Dveksler, G., Ruhl, S., Dieffenbach, C.W., and Pluznik, D. (1990) IL-6 induction of differentiation and 2'5' oligoadenylate synthetase in the murine macrophage cell line M-1. Cytokine 2:1-9.
3. Majde, J., Brown, R.K., Jones, M.W., Dieffenbach, C.W., Maitra, N., Krueger, J.M., Smitka, C.W., and Maassab, H.F. (1991) Viral toxicity: influenza virus-associated double-stranded RNA (dsRNA) from lung induces fever and sleep in rabbits. Microb. Pathogen. ACCEPTED FOR PUBLICATION.
4. Svetic, A., Finkelman, F.D., Dieffenbach, C.W., Scott, D.E., Steinberg, A.D. and Gause, W.C. (1991) Cytokine gene expression following primary immunization with GaM $\delta$  antibody. J. Immunol. ACCEPTED FOR PUBLICATION.
5. Abstracts to the American Society for Virology meeting, 1990. The society does not publish the abstracts.

ANNUAL PROGRESS REPORT

GRANT #: N00014-88-K-0337

R&T CODE: 4414812

PRINCIPAL INVESTIGATOR: David B. Hoyt, M.D.

INSTITUTION: University of California, San Diego

GRANT TITLE: Cellular Mechanism of an Immunologically Active  
Trauma Peptide

REPORTING PERIOD: 1 July 1990 - 30 June 1991 (12 months)

AWARD PERIOD: 1 July 1988 - 30 June 1991

OBJECTIVE: To further purify, characterize and evaluate the mechanism of immune suppression of a suppressive active peptide(SAP) by evaluating the mechanism of suppression on lymphocyte function.

ACCOMPLISHMENTS (last 12 months): During the last twelve months the mechanism of action on lymphocyte function has been better characterized and the peptide has been better purified.

T lymphocyte membrane receptor changes have been measured following PHA activation. The most notable inhibition on lymphocyte surface antigen expression was on the interleukin-2 receptor. Decreased excretion of IL-2 by human T lymphocytes was demonstrated as well. Human T lymphocytes exposed to SAP alone and in the presence of indomethacin, anti PGE<sub>2</sub> antibody, and IL-2 suggest that reconstitution with IL-2 or removal of inhibitory PGE<sub>2</sub> is insufficient to explain the mechanism of action. IL-2 biosynthesis can be partially reversed by addition of the Ca<sup>++</sup> ionophore A<sub>23187</sub> suggesting that SAP effects intracytosolic [Ca<sup>++</sup>]<sub>i</sub> signaling. Several sites on the T cell activation cascade are dependent on calcium. SAP exposure to human lymphocytes results in decreased inositol turnover (IP<sub>3</sub>). Bypassing protein kinase activation does not completely restore activity suggesting later sites are effected as well. Preliminary studies reveal SAP exposed cells have a rapid and sustained influx of calcium to supraoptimal levels. The influx can be partially reversed by pretreatment with calcium channel blockers.

SIGNIFICANCE: A leading cause of morbidity and death following injury is related to infection, sepsis and multiple organ failure. Circulating suppressor substances following injury and systemic inflammation contribute to this suppression. They may inhibit cellular immune function by altering the [Ca<sup>++</sup>]<sub>i</sub> concentration and interfering with essential activation steps. The ability to modulate these suppressive effects could have a significant effect on reversing post traumatic immunosuppression and reducing post traumatic sepsis, multiple organ failure and its associated morbidity and mortality.

WORK PLAN (next 12 months): Detailed mechanisms behind the regulatory roll of SAP are not clear, however, preliminary data suggests that SAP



interferes with the calcium signal in T cells which is an essential step in the activation of T cells. The elevation of intracellular free calcium to a precise level is a prerequisite for induction of T cell blastogenesis. The appreciation that transmembrane calcium signaling is exquisitely dependent on pH and sodium content requires that a purification scheme be developed to provide salt free SAP in order to avoid inappropriate conclusions regarding the mechanism of action. With this in mind we plan to develop an isolation procedure to provide salt free preparations of SAP for use during subsequent evaluation of the mechanisms of action by using a Bio-Gel P-2 size exclusion column (Bio-Rad, Richmond, CA). Suppressive fractions will then be applied to a TSK HW40 f size exclusion column. This size exclusion medium allows use of milli-Q-water as a mobile phase without significant loss in resolution. Salt-free SAP can be used in all subsequent analyses of mechanism of action. We will determine whether SAP mediated alterations in calcium signaling correlate with pathophysiologic inhibition of the T cell by evaluating suppression of PHA blastogenesis, the progression of early and late T-cell receptor expression, and IL-2 excretion. The association between deranged intra-cellular calcium signaling and each aspect of inhibition will be measured. The influence of SAP on the calcium signal will be modulated with calcium ionophores, calcium channel blockers and cytosolic release blockade (Dantrolene). The effects of these agents will be evaluated on a.) the intracellular  $Ca^{++}$  signal, b.) mitogen induced blastogenesis, c.) antigen expression during blastogenesis and d.) IL-2 excretion to define the origin of the derangement and whether modulation of immunosuppression caused by SAP is possible.

#### PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Hansbrough JF, Zapata-Sirvent R, Hoyt DB: Postburn immune suppression: An inflammatory response to the burn wound? J Trauma 30:671-675, 1990.
2. Tompkins SD, Gregory S, Hoyt DB, Ozkan AN: In vitro inhibition of IL-2 biosynthesis in activated human peripheral blood mononuclear cells by a trauma-induced glycopeptide. Immunology Letters 23:205-209, 1990.
3. Hoyt DB, Ozkan AN, Hansbrough JF, Marshall L: Head injury: An immunologic deficit in T-cell activation. J Trauma 30:759-767, 1990.
4. Hoyt DB, Ozkan AN: Immunosuppression in trauma patients. Journal of Intensive Care Medicine 6:71-90, 1991.
5. Hoyt DB, Ozkan AN, Frevert J, Junger W, Loomis W: Mechanisms of alteration in  $Ca^{2+}$  homeostasis by a trauma peptide causing immunosuppression. Journal of Surgical Research (in press).
6. Hoyt DB: Immunoregulation by the extracellular matrix following injury. 2nd International Congress on the Immune Consequences Proceedings: 7, 1991. (Abstract)
7. Hoyt DB: Circulating immuno-suppressive mediators, mechanisms of action. 2nd International Congress on the Immune Consequences Proceedings: 15, 1991. (Abstract)

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1896

R&T CODE: 4414032

PRINCIPAL INVESTIGATOR: G. Miller Jonakait

INSTITUTION: Rutgers University

GRANT TITLE: Effect of Immune Cytokines on Injured Sympathetic Ganglia

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 June 1990 - 31 May 1993

OBJECTIVE: To test the hypothesis that the interleukin-1-induced increase in sympathetic substance P (SP) constitutes one part of a neural-immune interaction that facilitates regeneration of the injured nervous system.

ACCOMPLISHMENTS (last 12 months): We have made progress on a number of fronts. We have determined by Northern blot analysis that IL-1-induced increases in SP are secondary to increases in mRNA coding for the preprotachykinin (PPT) precursor of SP. Moreover, nuclear transcription assays showed this to be an effect on PPT transcription itself. Further studies showed that depolarizing agents, interferon- $\gamma$ , glucocorticoid hormones, and prostaglandin synthesis inhibitors diminished the induction of SP and PPT mRNA by IL-1.

We have examined the ability of macrophage-produced cytokines other than IL-1 to raise SP in cultured sympathetic ganglia. We have found that IL-6 does not raise SP at any of the various concentrations tested, but tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) more than doubles SP. Moreover, we have found that lipopolysaccharide (LPS), at concentrations as low as 1 ng/ml, dramatically raises SP and works synergistically with the T cell product IL-4 to raise SP still further. The effect of LPS is only partly mediated by an LPS induction of IL-1.

We have also developed dissociated cell culture for both sympathetic ganglia and for CNS nuclei, perfected the surgery required to deafferent superior cervical ganglia in vivo, and have established immunocytochemistry for the detection of SP in lymph nodes.

SIGNIFICANCE: These studies should provide greater insight into the role played by the immune system in facilitating recovery following nervous system injury, and will provide an

understanding of the role played by the injured nervous system in triggering that immune system response.

WORK PLAN (next 12 months): As our tools are now in place, we plan now to determine whether IL-1-induced increases in ganglionic SP result in concomitant increases in terminal SP, particularly in those terminals innervating lymphoid organs; determine whether IL-1 promotes proliferation of cells (and determine which cells) in dissociated cultures; investigate further the synergy between LPS and IL-4 (is it, for example, due to the LPS induction of IL-1?); and investigate possible synergy with other T cell products.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Hart, R.P., A. Shadiack, and G. Miller Jonakait (1991). Substance P gene expression is regulated by interleukin 1 in cultured sympathetic ganglia. *J. Neurosci. Res.*, in press.
2. Jonakait, G. Miller, S. Schotland, and R.P. Hart (1991). Effects of lymphokines on substance P in injured ganglia of the peripheral nervous system. In *Substance P and Related Peptides: Cellular and Molecular Physiology*, Eds./ S. Leeman, J. Krause, F. Lembeck. *Ann. N.Y. Acad. Sci.*, in press.
3. Shadiack, A.M., D. Ganea, R.P. Hart and G.M. Jonakait (1991). Immunoactive agents stimulate substance P in cultured sympathetic ganglia. Abstract, Soc. for Neuroscience, in press.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1637

R&T CODE: 4414025

PRINCIPAL INVESTIGATOR: Martin Lotz, M.D.

GRANT TITLE: Interactions between neuropeptides and cytokines in host responses to injury

REPORTING PERIOD: 1 April 1990 - 30 April 1991 (12 months)

AWARD PERIOD: 1 April 1990 - 30 April 1993

OBJECTIVE: To Examine effects of neuropeptides on cells of the immune system, to study production of neuropeptides by cells that participate in host responses to injury and to analyze the effects of factors released from injured tissue on immune function.

### ACCOMPLISHMENTS (last 12 months):

- (i) Met-enkephalin expression in the immune system and in connective tissues.

In vitro activated human blood monocytes and lymphocytes expressed preproenkephalin mRNA. Monocytes, but not T lymphocytes contained intracellular met-enkephalin immunoreactivity and secreted met-enkephalin. Both cell types secreted enkephalin precursor proteins. Met-enkephalin immunoreactivity was also detected by staining of lymphoid tissues.

In a subsequent study it was found that human articular chondrocytes express the prepro-enkephalin gene, process the precursor proenkephalin A into mature met-enkephalin and that expression of this neuropeptide is related to activation and proliferation of these cells. Prepro-enkephalin gene expression was inducible by serum factors. Proliferating chondrocytes expressed high levels of prepro-enkephalin mRNA, but it was not detectable in confluent, contact-inhibited cells. The two growth factors TGF $\beta$  and PDGF, phorbol-myristate-acetate and dibutyrate cyclic-AMP, but not dexamethasone, increased prepro-enkephalin mRNA. Primary chondrocytes cultured in the presence of transforming growth factor  $\beta$  developed cartilage nodules and this was associated with high levels of prepro-enkephalin mRNA. In contrast, cartilage nodules and the expression of enkephalin were suppressed by interleukin-1 $\beta$ . Chondrocytes store met-enkephalin intracellularly and secrete this neuropeptide after growth factor stimulation.

- (ii) TGF $\beta$  and monocyte function

This study analyzes changes in cell surface marker expression and cytokine production during in vitro differentiation of human blood monocytes and defines the effects of TGF $\beta$  in this process.

Fresh blood monocytes express low levels of CD16/FcRIII (0-20%) and this increases to approximately 40% after 7 days in culture. High levels of IL-1 $\beta$  and IL-6 are produced by blood monocytes following stimulation with LPS. In vitro derived macrophages, however, release only 46% and 31% of these cytokines. The addition of exogenous TGF $\beta$  to monocytes leads to a rapid increase in CD16 expression and within 24 h reduces the titers of LPS-induced IL-1 $\beta$  and IL-6 to levels as low as those seen in day 7 macrophages. By *in situ* hybridization and Northern blotting it was shown that in contrast to the decrease in the protein levels of these interleukins, TGF $\beta$  did not detectably alter their mRNA expression. These in vitro studies were validated on mononuclear phagocytes from synovial fluids which were exposed to TGF $\beta$  in vivo.

(iii) Regulation of TGF $\beta$  expression in chondrocytes  
TGF $\beta$  represents a group of growth factors that modulate chondrocyte function in development and in responses to injury. We showed that chondrocytes produce biologically active TGF $\beta$ , studied the regulation of TGF $\beta$  gene expression and showed that the different TGF $\beta$  isoforms can function as endogenous cartilage regulatory factors.

#### SIGNIFICANCE:

The findings that human articular chondrocytes express the prepro-enkephalin gene, process the propeptide and secrete mature met-enkephalin suggest that enkephalins can function as regulatory factors among non-neuronal cells, contribute to joint inflammation and possibly to homeostasis and remodeling of connective tissues.

The studies on TGF $\beta$  and monocyte function show that TGF $\beta$  induces qualitatively similar changes in monocytes as observed during in vitro differentiation. The production and activation of TGF $\beta$  by mononuclear phagocytes indicates that this cytokine can participate in the regulation of monocyte differentiation. Phenotype and function of monocytes is similar when exposed to TGF $\beta$  in vitro or in vivo. This TGF $\beta$  effect on monocyte function is consistent with the function of this cytokine in protecting the organism from inflammatory damage during responses to injury.

The ability of chondrocytes to produce TGF $\beta$  is an important component in the regulation of cartilage responses to injury since TGF $\beta$  directly influence chondrocyte functions and modulate the effects of other growth factors.

WORK PLAN (next 12 months): In the studies on enkephalins we will determine how these neuropeptides can regulate functions of immune and connective tissue cells. In these experiments enkephalins will be added to cell cultures, degradation of endogenously produced met-enkephalin will be prevented with enkephalinase inhibitors and the expression of endogenously produced enkephalins will be blocked with antisense oligonucleotides.

In the work on TGF $\beta$  we will use a similar approach with antisense oligonucleotides to determine the role of endogenously produced TGF $\beta$  in the regulation of monocyte and chondrocyte function.

#### PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Kuis, W., Villiger, P., Leser, H.G., Lotz, M. Differential processing of proenkephalin-A by human peripheral blood monocytes and T lymphocytes. J. Clin. Invest. in press.

2. Guerne, P.A., Lotz, M. Interleukin-6 and transforming growth factor  $\beta$  synergistically stimulate chondrosarcoma cell proliferation. J. Cell Phys. in press.

3. Lotz, M., Villiger, P. Neuroimmune interactions in homeostasis and pathophysiology of connective tissues. EBSCO Media, in press.

4. Villiger, P., Lotz, M. Differential expression of TGF $\beta$  isoforms by human articular chondrocytes and function as endogenous cartilage regulatory factors. submitted.

5. Leser, H.-G., Villiger, P., Lotz, M. TGF $\beta$  and monocyte differentiation. Effects on surface marker expression and cytokine production. submitted.

6. Villiger, P., Moats, T., Kuis, W., Lotz, M. Expression and processing of preproenkephalin by human articular chondrocytes. submitted.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1547

R&T CODE: 4414023

PRINCIPAL INVESTIGATOR: Matthew J. Kluger, Ph. D.

INSTITUTION: University of Michigan

GRANT TITLE: Role of Cytokines in the Acute Phase Response

REPORTING PERIOD: 1 January 1990 - 1 May 1991

AWARD PERIOD: 1 January 1990 - 31 December 1992

OBJECTIVE: The purpose of these studies is to assess in an animal model the roles of the cytokines IL-1, IL-6, TNF, and IFN $\alpha$  and  $\beta$  in anorexia/cachexia induced by an acute infection.

ACCOMPLISHMENTS (last 12 months): We have characterized the temperature, activity, food intake, water intake, and body weight changes of mice to various doses of influenza virus. After testing about 7 doses of virus, two doses have been selected ( $1.4 \times 10^2$  PFU and  $5.5 \times 10^4$  PFU) for more extensive investigation relating cytokine levels to the observed changes in physiology and behavior. Lung lavage fluid, fluid from freeze-thawed cells isolated from lung lavage fluid, and plasma are being assayed for presence of IL-6, TNF, and IFN $\alpha$ .

SIGNIFICANCE: Cytokines are being implicated as mediators in many of the physiological and behavioral manifestations of disease. It is not yet clear whether the acute phase responses, thought to be triggered by cytokines, are helpful or harmful to the infected host. Based on the long evolutionary history of fever, survival studies, and assessment of the effects of temperature on immune responses, I and others have argued that fever is adaptive. There are also data supporting the hypothesis that anorexia and other behavioral manifestations that occur with disease are adaptive. By reversing the physiological and behavioral manifestation of disease (e.g. by use of specific antibodies) will this increase mortality and morbidity? The experiments outlined in this proposal will (1) characterize the cytokine profiles in influenza-infected mice, (2) assess whether many of the behavioral manifestations of this disease are caused by these cytokines, (3) allow us to assess the in vivo interrelationships among cytokines, and perhaps most importantly (4) set the stage for future studies to assess the adaptive significance of these behavioral acute phase responses.

WORK PLAN (next 12 months):

1. To determine whether the observed decline in body temperature in mice inoculated with influenza virus is due to a fall in thermoregulatory "set-point".
2. To continue our studies to characterize the plasma and lung profiles of the cytokines IL-1, IL-6, TNF, IFN $\alpha$  and IFN $\beta$  of mice inoculated with various doses of influenza virus.
3. To begin using various inhibitors of cytokine action (e.g. IL-1 receptor antagonist protein from Upjohn Co.; or antiserum to TNF, IL-1 $\beta$ , IL-6) to determine whether this alters the physiological and behavioral changes observed in influenza-infected mice.
4. To begin to assess whether treatment of influenza-infected mice with those agents mentioned in # 3 (above) will alter their morbidity and mortality rate.

PUBLICATIONS AND ABSTRACTS (last 12 months):

None related to these studies. We anticipate preparing an abstract that will be submitted to the Third International Cytokine Workshop this month, and a manuscript for publication within the next few months.

# ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1130

R&T CODE: 4414820

PRINCIPAL INVESTIGATOR: Elizabeth J. Kovacs, Ph.D.

INSTITUTION: Loyola University, Stritch School of Medicine

GRANT TITLE: Control of Cytokine Gene Expression in Macrophages:  
Role of Second Messenger Pathways

REPORTING DATE: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 November 1988 - 31 October 1991

OBJECTIVE: The overall objective of the proposed research is to enhance our understanding of the intracellular second messenger pathways involved in the regulation of the expression of soluble cellular mediator (cytokine) genes in macrophages by bacterial lipopolysaccharide (LPS) and interleukin-2 (IL-2).

ACCOMPLISHMENTS (last 12 months): The culture of peritoneal macrophages with IL-2 selectively induces the expression of PDGF A chain and B chain mRNAs but not TGF $\beta$ . These data differ from those of our earlier work performed in human PBL and purified blood monocytes. In contrast to peritoneal macrophages in which TGF $\beta$  mRNA is constitutively expressed and cannot be upregulated, it is not spontaneously expressed in PBL yet can be induced within 2 hours of IL-2 treatment. Both non-adherent and adherent populations of PBL can be induced by IL-2 to express TGF $\beta$  mRNA. Like PBL, blood monocytes express TGF $\beta$  mRNA following IL-2 treatment, however the peak levels of expression are not achieved until 18 hours. In contrast to TGF $\beta$  mRNA which can be induced in both adherent and non-adherent PBL, only the adherent population of PBL expressed the PDGF mRNAs. Neither adherent PBL nor blood monocytes spontaneously expressed PDGF mRNAs but the messages can be induced following treatment with IL-2. It is likely that the different patterns of expression of the PDGF and TGF $\beta$  genes in peritoneal macrophages and PBL can be attributed to the states of differentiation and activation of the cells. Immunoblot assays for PDGF A chain and B chain proteins were developed to allow more efficient detection of production of these cytokines.

Studies designed to block the effects of fibrogenic mediators (TGF $\beta$  and PDGF) on fibroblast proliferation have been completed. One set of these studies involves the antiproliferative effects of heparan sulfate proteoglycan (HS). HS was shown to inhibit the proliferation of fibroblasts and smooth muscle cell stimulated with medium supplemented recombinant TGF $\beta$  or PDGF. The continuous presence of HS was not necessary for growth inhibition, suggesting that the inhibitory effect of HS on cell proliferation is not merely due to interaction of HS with growth factors. We cannot rule out the possibility that HS plays a role in the regulation of cell surface molecules involved in substrate attachment or growth factor signal transduction. Glycosaminoglycans, including heparin and heparan sulfate, failed to inhibit cytokine (IL-1 $\beta$  and TNF $\alpha$ ) gene expression in macrophages stimulated with LPS or IL-2.

Studies involving an examination of the role of cAMP on the expression of cytokine genes in the ANA-1 murine macrophage cell line reveal that there are two classes of cytokine genes ones which require increased intracellular levels of cAMP to be induced and ones which do not. Expression of PDGF B chain mRNA is induced by treatment with cAMP inducers while IL-1 $\alpha$ , IL-1 $\beta$  and TNF $\alpha$  are not.

A calcium/calmodulin (CAM) kinase-dependent pathway is involved in the control of PDGF A chain mRNA expression in the monocyte cell line THP-1.



WORK PLAN (next 5 months): Continue work on the antiproliferative effects of fibrogenic mediators on fibroblast proliferation of heparan sulfate proteoglycan. This will include an analysis of second messenger pathways involved in mediating the growth inhibitor effect of heparan.

Additional studies will include the examination of the control of expression of cytokine genes in the ANA-1 cell line in response to TNF $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$ . A comparison of the stimuli needed to induce the expression of TNF $\alpha$  and PDGF A chain mRNA expression in the human monocyte cell line THP-1 will be made.

Further analysis of the expression of fibrogenic cytokines in human peripheral blood monocytes will be performed. An analysis of IL-2 and LPS induced second messenger pathways leading to the expression of PDGF and TGF $\beta$  mRNAs will be made.

PUBLICATIONS (last 12 months):

1. Publications

- Kovacs, E.J., B. Brock, I.E. Silber, and J.E. Neuman. (1990) LAK cell expression of PDGF and TGF- $\beta$  mRNAs. (eds, M.C. Podwanda, J.J. Oppenheim, M. Kluger, and C.A. Dinarello), Molecular and Cellular Biology of Cytokines, New York, Alan R. Liss, pp. 407-412.
- Kovacs, E.J. (1991) Control of IL-1 and TNF mRNA expression by inhibitors of second messenger pathways. (ed, E. Kimball), Cytokines in Inflammatory Diseases. Boca Raton, FL, CRC Press, pp. 87-105.
- Kovacs, E.J. (1991) Fibrogenic cytokines: The role of immune mediators in the development of fibrosis. Immunology Today, 12:17-23.
- Kovacs, E.J. and J.E. Neuman. (1991) Selective induction of PDGF A chain and B chain gene expression in rat peritoneal macrophages by interleukin-2, (eds, M.M. Meltzer and A. Montovani), Cellular and Cytokine Networks in Tissue Immunity, New York, Wiley. In press.
- Kovacs, E.J., B. Brock, I. Silber, and J.E. Neuman. Fibroblast proliferation and connective tissue production by cytokines derived from IL-2-treated peripheral blood leukocytes: expression of TGF $\beta$  and PDGF A chain genes. Submitted to J. Clin. Invest. for publication.
- Silber, I.E., J.M. Walenga, J. Fareed, and E.J. Kovacs. Mechanism(s) responsible for the control of cell proliferation by heparan sulfate. Submitted to J. Leukocyte Biol. for publication.
- Kovacs, E.J. and J.E. Neuman. Expression of PDGF genes in peritoneal macrophages: selective induction of expression by interleukin-2. Submitted to J. Leukocyte Biol. for publication.
- Kovacs, E.J. and J.R. Frazier-Jessen. Overview: The inflammatory process. (eds, L.B. Schook and D.L. Laskin), Xenobiotic induced inflammation: Roles of cytokines and growth factors. Orlando, FL, Academic Press. In preparation.
- Kovacs, E.J. Selective induction of expression of PDGF B chain mRNA in the ANA-1 murine macrophage cell line by cAMP. In preparation.
- Kovacs, E.J. and S. Van Stedum. Control of expression of PDGF A chain mRNA by calcium-calmodulin kinase. In preparation.

2. Invited Presentations

- "Control of PDGF and TGF $\beta$  gene expression." Department of Medicine, Brown University, Providence RI, Oct 1990.
- "Selective induction of PDGF A chain and B chain gene expression in rat peritoneal macrophages by interleukin-2." International RES Congress, Crete, Greece, Oct 1990.
- "Role of PDGF and TGF $\beta$  in the development of peritoneal adhesions." Fifth Annual Conf on Clinical Immunology, Chicago, IL, Nov, 1990.
- "Control of expression of PDGF A chain and B chain genes." Biological Response Modifiers Program, NCI-FCRDC, Frederick, MD, February 1991.
- "Role of cytokines in the control of connective tissue production." Department of Pathology, Univ of Kansas, Kansas City, KS, March 1991.
- "Cytokines." University Rheumatology Council, Chicago IL, May 1991.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1895

R&T CODE: 4414026

PRINCIPAL INVESTIGATOR: David R. Knighton, M.D.

INSTITUTION: University of Minnesota

GRANT TITLE: Microenvironmental Control of Monokine Production  
in Wound Repair

REPORTING PERIOD: 1 June 1990 - 31 May 1991 (12 months)

AWARD PERIOD: 1 June 1990 - 31 May 1993

OBJECTIVE: To investigate the effect of the wound space oxygen, lactate, and pH microenvironment on the regulation of monokine production and to determine the interrelationship between these different environmental conditions and known biochemical modulators of macrophage function on monokine production. Utilizing a computer-controlled, constant perfusion, tissue culture system (Opticell) which allows for precise control of the cellular environment, we will determine the effect of various environmental and biochemical parameters on macrophage production of platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor  $\beta$  (TGF $\beta$ ), transforming growth factor  $\alpha$  (TGF $\alpha$ ), tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin-1 (IL-1), interleukin-6, and macrophage derived angiogenesis factor (MDAF).

ACCOMPLISHMENTS (last 12 months): In the first twelve months of work on this project we have overcome some major obstacles and refined the system, confirmed our original preliminary results, and begun work on the interrelationship of oxygen tension and  $\gamma$ -interferon on the control of macrophage monokine production. In our initial studies the peripheral blood monocytes were seeded into the system using DMEM and 15% human serum. This was done in order to be consistent with our previous work in both static tissue culture and the early work with the Opticell system. Two problems arose during these studies. It proved to be very difficult and expensive to remove all of the serum from the system when the switch was made to serum-free media (HL-1). This switch to HL-1 and complete removal of serum is necessary because serum in the culture supernatants to be tested for angiogenesis will result in false positive results. In addition, our supplier of human serum (Gibco) had to recall a lot of serum due to potential contamination with HIV virus and was subsequently unable to ship any human serum for some time. This resulted in the loss of one run and the loss significant time while awaiting new

serum. These two factors forced us to seed the monocytes into the system using HL-1 right from the start of the run. This resulted in our having to refine the seeding and conditioning phases of the Opticell runs.

Studies have begun examining the interrelationship between oxygen tension (2% or 20%) and  $\gamma$ -interferon ( $\gamma$ IFN). The initial studies in this series of experiments were performed with monocytes seeded into the system in human serum. The results of these studies indicated that TNF $\alpha$  production increased under conditions of hypoxia and that this increase was blocked by  $\gamma$ IFN. Measurable amounts of TGF $\beta$  and nitrous oxide (as nitrates/nitrites) were produced, but did not seem to be affected by the presence or absence of hypoxia and  $\gamma$ IFN. No measurable amounts within the limits of detection were found for PDGF, IL-1 or IL-6. Angiogenesis activity was found in the hypoxic (2%) conditioned media, was absent in the control (20% O<sub>2</sub>), the  $\gamma$ -IFN at 20% O<sub>2</sub> and in the  $\gamma$ IFN at 2% O<sub>2</sub>.

SIGNIFICANCE: These results would indicate that  $\gamma$ IFN may modulate the effect of hypoxia on macrophage monokine production. In particular,  $\gamma$ IFN may down regulate angiogenesis factor production by macrophages.

WORK PLAN (next 12 months): The specific objectives for the next year will be to investigate the effects of the various microenvironmental parameters alone, in combination, and in combination with  $\gamma$ IFN on monokine production. These studies will now be performed using only HL-1 culture media, peripheral blood monocytes/macrophages, and the other system conditions we have optimized during the past year.

PUBLICATIONS AND ABSTRACTS (last 12 months): None.

# ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1069

R&T CODE: 4414720

PRINCIPAL INVESTIGATOR: James M. Krueger

INSTITUTION: University of Tennessee, Memphis

GRANT TITLE: Immunophysiology: Central Mechanisms of the Acute Phase Response

REPORTING PERIOD: 1 July 1990 - 31 May 1991

AWARD PERIOD: 1 December 1989 - 30 November 1992

OBJECTIVE: The overall objective is to examine the regulation of the acute-phase response (APR) by central nervous system (CNS) mechanisms and to determine primary and secondary signals that indicate these responses.

ACCOMPLISHMENTS: Changes in sleep occurring over the course of bacterial and fungal infections have only recently been documented, but virally-induced changes in sleep have not yet been investigated. We investigated the effects of viral challenge on sleep, and determined whether virally-induced physiological tolerance involves sleep as well as pyrogenic responses after repeated viral challenge. We also investigated the role of double-stranded (ds)RNA in initiating physiological responses to virus.

Two groups of males in New Zealand White rabbits were implanted with EEG electrodes and a brain thermositor. All rabbits were inoculated intravenously at 9:00 a.m. on three consecutive days. Group I received: day 1, 1 ml of control egg allantoic fluid diluted in Hanks' balanced salts; day 2, influenza virus (20,000 mouse LD<sub>50</sub> of A/PR/8/34); day 3, viral challenge as in group I on day 2. Group two received: day 1, 1 ml of pyrogen-free saline solution; day 2, synthetic dsRNA (poly I:C, 2.5 ug/kg); day 3, viral challenge as in group I on day 2. Sleep-wake activity and brain temperatures (T<sub>br</sub>) were continuously recorded throughout the 72 h experimental period.

In group I, the first injection of the virus significantly enhanced NREMS and EEG slow-wave amplitudes and elicited fever (increased T<sub>br</sub>) during 4-8 h postinjection. The viral challenge caused little change in REMS. By hours 10-24 h postinjection, sleep and T<sub>br</sub> values were close to day 1 (baseline) values. On day 3, viral challenge failed to alter sleep or T<sub>br</sub>. The time courses of effects of poly I:C on enhanced sleep and T<sub>br</sub> were slightly different from virally-induced effects, and poly I:C inhibited REMS. Nevertheless, viral inoculation did not induce NREMS or fever after the poly I:C challenge.

It is clear, therefore, that challenge with influenza virus or poly I:C induced enhanced sleep, fever and physiological tolerance. Rabbits neither slept more nor fevered if challenged with the virus 24 h after the first challenge with the virus or poly I:C, which suggest the involvement of dsRNA in physiological tolerance. We hypothesize that virally-induced sleep and tolerance are mediated through cytokines (perhaps interferon) induced by dsRNA.

SIGNIFICANCE: Our sleep data are the first to be obtained after a viral challenge. They suggest that viral double-stranded RNA drives sleep and fever responses via induction of systematic interferon production. Finally, these data show for the first time that virally-induced physiological tolerance involves sleep as well as fever.

WORK PLAN: The specific plans for next year include: 1) Rabbits immunized against poly I:C and demonstrated to have neutralizing antibodies will be challenged with influenza virus or poly I:C and their somnogenic, pyrogenic responses measured. 2) Sleep changes expressed during a full-blown infection with influenza virus in a susceptible species, the mouse, will be evaluated.

PUBLICATIONS AND REPORTS (last 12 months):

A) Refereed manuscripts

1. Johannsen, L., J. Wecke, F. Obal, Jr., and M. Krueger. Macrophages and pyrogenic muramyl peptides during the digestion of staphylococci. Am. J. Physiol. 260: R126-R133, 1991.
- 2) Obal, Jr., F., M. Opp, A.B. Cady, L. Johannsen, A.E. Postlethwaite, H.M. Poppleton, J.M. Seyer, and J.M. Krueger. Interleukin-1 $\alpha$  and an interleukin-1 $\beta$  are somnogenic. Am. J. Physiol. 259:R439-R436, 1990.
- 3). Krueger, J.M., M. Opp, L.A. Toth, and L. Kapas. Immune regulation, hormones, and sleep. In: Sleep '90, J. Horne (ed.) Pontenagel Press, Bochum, pp. 371-374, 1990.
- 4) Majde, J.A., R.K. Brown, M.W. Jones, C.W. Dieffenbach, N. Maitra, J.M. Krueger, A.B. Cady, C.W. Smitka, and H.F. Maassab. Detection of toxic viral-associated double-stranded RNA (dsRNA) in influenza-infected lung. Microbial Pathogenesis (in press).
- 5) Opp, M.R., and J.M. Krueger. An interleukin-1 receptor antagonist blocks interleukin-1-induced sleep and fever. Am. J. Physiol. (in press).
- 6) Kapas, L., L. Payne, F. Obál, Jr., M. Opp, L. Johannsen, and J.M. Krueger. Sleep in diabetic rats: effects of interleukin 1. Am. J. Physiol. (in press).
- 7) Johannsen, L., H. Labischinski, and J.M. Krueger. Somnogenic activity of pseudomurein in rabbits. Infection and Immunity (in press).

B) Reviews

- 1) Krueger, J.M., M.R. Opp, L. Kapas, M. Kimura-Takeuchi, and L. Toth. Muramyl peptides and interleukin-1 in sleep regulation. In: Endocrine and Nutritional Control of Basic Biological Functions. R. Murison, et al. (eds) Hogrefe and Huber Publishers.
- 2) Toth, L.A., and J.M. Krueger. Infectious disease, cytokines and sleep. In: The Diencephalon and Sleep; M. Mancina, editor, Raven Press, NY. 1990, pp. 331-341.
- 3) Krueger, J.M., and F. Obál, Jr. Sleep Factors. In: Sleep and Breathing, N.A. Saunders and C.E. Sullivan (eds). Marcel Dekker, Inc. (submitted).

# ANNUAL PROGRESS REPORT

GRANT # : N00014-89-J-18C3

R&T CODE: 4415911

PRINCIPAL INVESTIGATOR: Dr. Louis Marzella

INSTITUTION: University of Maryland School of Medicine, Department  
of Pathology

GRANT TITLE: Effects of Pressure on Intravascular Adhesion  
Molecules

REPORTING PERIOD: 1 June 1990 - 31 May 1991 (12 months)

AWARD PERIOD: 1 May 1989 - 30 April 1992

OBJECTIVE: Determine if changes in pressure cause: increased expression of adhesion molecules on vascular endothelium, altered blood flow and organ damage characteristic of decompression sickness.

ACCOMPLISHMENTS (last 12 months): We have completed the characterization of organ blood flows by the radiolabelled microsphere technique in a rat model of decompression sickness. The rats were dived with compressed air in a steel hyperbaric chamber. Rats in the experimental group were dived to a depth of 198 feet of sea water (fsw) at a compression rate of 0.33 fws/sec and a decompression rate of 3.3 fsw/sec; total bottom time was 71 min. Rats in the control group were dived to 165 fsw and were decompressed at 0.27 fsw/sec with a 10 min stop at 100 fsw. The control rats showed no physiologic or pathologic changes. Rats in the experimental group showed gait disturbances and a 44 percent mortality. In this group 10 min after the dive the blood flow increased in the spinal cord (20 percent, Fig. 1A) and decreased in both lung (40 percent, Fig 2 A) and non-respiratory muscle (50 percent, Fig. 3)  $P < 0.05$ . At 1 and 3 hr after the dive, the blood flow to these organs returned to normal. Beginning at 1 hr, significant increases in blood flow were observed in liver, spleen, small intestine, and cecum (Fig. 4). Increased splanchnic circulation was also observed at 3 hr. Analysis of fractional blood flow distribution showed that the percentage of cardiac output reaching the spinal cord (Fig. 1B) and the splanchnic organs (not shown) increased. On the other hand, the percentage of cardiac output reaching the muscle underwent a compensatory decline.

The most likely explanation for the decreased blood flows to the spinal cord and GI tract is that they are caused by focal ischemia due to microvascular dysfunction. We have focused on the CNS to attempt to identify the mechanism of the postulated microvascular dysfunction. We found no evidence of extravascular accumulation of nitrogen bubbles in myelin-rich regions of the cord. A quantitative analysis of space-occupying lesions in the white matter of the cord failed to show autochthonous gas (Table 1). We also investigated the possibility that damage to the blood-brain barrier could be the cause of microvascular dysfunction. To this end the integrity of the barrier was probed with monoclonal antibodies to GFAP. By immunocytochemical criteria no

disruption of astrocytic perivascular processes could be demonstrated 10 min after decompression (Fig. 5)

Activation of acute inflammatory responses could contribute to microvascular dysfunction. To evaluate this possibility we examined the total levels of complement in the plasma. Table 2 shows that a slight but significant decline in total complement occurred 10 min after the dive. It is likely that activation of specific complement components occurs systemically after decompression in this model. Preliminary determinations of serum levels of tumor necrosis factor and interleukin-6 were inconclusive due to large variability (Tables 3,4).

Altered expression of adhesion molecules is another possible mechanism for microvascular dysfunction. We have used a horseradish peroxidase technique to examine the expression of ICAM-1 in the spinal cord of rats with decompression sickness. Enhanced immunoreactivity was found 4 hr after diving in the endothelium of the microcirculation. The expression of ICAM-1 had returned to baseline at the next time-point examined (5 days).

SIGNIFICANCE: The pathophysiology of decompression sickness appears to be explainable by focal ischemia in the spinal cord and by activation of adhesion molecules in the microcirculation of the cord. If this mechanism can be confirmed, new approaches for the treatment of decompression system will be developed.

WORK PLAN (next 12 months): The focus of the work will be to confirm, quantify and determine the kinetics of increased expression of adhesion molecules in the microvasculature of the spinal cord. We will also test other known target organs of decompression sickness for increased expression of these molecules.

PUBLICATIONS AND ABSTRACTS (last 12 months)

1. Marzella L., Yin A., Muhvich K.H., and Myers R.A.M. (1991) Organ blood flows and central hemodynamics in a rat model of decompression sickness. Undersea Biomed. Res. 18 : 72-73.

2. Marzella L., Yin A., and Myers R.A.M. (1991) Morphometric assessment of gas bubbles in the spinal cord of rats with decompression sickness. Undersea Biomed. Res. 18 : 73.

# ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1580

R&T CODE: 4414024

PRINCIPAL INVESTIGATOR: C. Richard Parker, Jr.

INSTITUTION: University of Alabama at Birmingham

GRANT TITLE: The Immune-Adrenal Axis in Humans

REPORTING PERIOD: 1 March 1990 - 28 February 1991

AWARD PERIOD: 1 March 1990 - 28 February 1993

OBJECTIVE: Define the effects of cytokines on growth and steroidogenesis in the human adrenal cortex in vitro and explore the relationship between circulating cytokines and adrenal steroidogenesis in healthy and stressed humans.

ACCOMPLISHMENTS (last 12 months): The effects of recombinant IL-1 $\beta$  on adrenal production of its major secretory products, i.e. cortisol (F) and dehydroepiandrosterone sulfate (DS), have been studied. Adrenal glands were obtained at the time of autopsy, usually 0.5-5 hours postmortem. To address the effects of IL-1 on the whole adrenal, we minced the adrenal into  $\approx$  2-3 mm<sup>3</sup> pieces and used the fragments for organ culture or else prepared isolated cells. Cells were suspended in culture medium (McCoy's 5A containing 5% FBS, 26 mM sodium bicarbonate, 10 mM hepes, antibiotics, and an antimycotic) and plated (usually 3-12.5  $\times$  10<sup>5</sup>/well).

To address the issue of whether IL-1 might selectively affect one of the adrenal cortical zones, the adrenal capsule and adherent cells (F is the quantitatively major steroid produced by these outer zone cells) was dissected from the inner zone tissue (which produces androgens, especially DS). The inner and outer zone tissues were separately processed to yield cells. For organ cultures, tissues were exposed to the test reagents beginning on the first day. For cell culture (cells from whole adrenal or isolated zones), test reagents were usually added after cells were well established, i.e., 5-6 days after initiation of culture. All experimental conditions were conducted in replicates of 4-5. After culture, the media were assayed for DS and F, and cells or tissues were homogenized and assayed for protein.

In 2 organ culture studies DS production was high initially in all circumstances and declined in the absence of ACTH. IL-1 $\beta$  (5 U/ml) had no effect on DS secretion compared to that in control media (McCoy's + 10% human serum). Whereas ACTH (1  $\mu$ g/ml) increased DS production by day 3 or 4 of culture, IL-1 $\beta$  had no effect on the ACTH-stimulated DS production. F production was about 1/8 that of DS on day 1 of culture, regardless of culture media used. Thereafter, F levels declined except in the presence of ACTH (F increased 3-6 fold by day 3-4). IL-1 $\beta$  did not alter basal or ACTH-stimulated F.

In cultured whole adrenal cells, DS and F levels declined in the absence of ACTH. ACTH (1  $\mu$ g/ml) increased DS 8 fold and F over 100 fold during 5 days culture. IL-1 $\beta$  (5 U/ml) had no effect on basal or ACTH-stimulated DS or F production. Also, ACTH (0.1  $\mu$ g/ml) stimulated DS and F production, but IL-1 $\beta$  (0.5 or 0.05 U/ml) had no effect. Experiments using inner or outer zone cells yielded similar results.

Based on the above results of experiments conducted with varying doses of ACTH and IL-1 $\beta$  and with varying approaches, we suspect that IL-1 $\beta$  has its in vivo effects on adrenal steroidogenesis by acting on extra-adrenal sites, most likely the brain and/or pituitary gland. We have not, however, ruled out the possibility that fetal bovine serum or human serum, one of which was present in all of the above studies, may



have impaired or reversed any direct effects of IL-1 $\beta$  on the adrenal cortex.

For studies of TGF $\beta$  (porcine platelet-derived TGF $\beta$ 1) on cell proliferation, n=8 to date, cells from the whole adrenal or separated zones were established in the culture over the course of 3-6 days, at which time test reagents were added. After 3-4 days of exposure to experimental conditions, 3  $\mu$ Ci of [ $^3$ H] thymidine were added to each dish for 14-24 hrs pulse labeling. After washing the cells, we solubilized them and isolated the DNA by TCA precipitation. Our preliminary studies can be summarized as follows: TGF $\beta$ -1 (0.1 or 1.0 ng/ml) causes about a 50% reduction in [ $^3$ H] thymidine incorporation compared to control cultures. ACTH (0.1  $\mu$ g/ml) has no consistent independent effect but partially reverses the inhibitory effects of TGF $\beta$ . The effects of TGF $\beta$  (0.1 or 1.0 ng/ml) +/- ACTH (0.1  $\mu$ g/ml) on steroidogenesis in whole adrenal cells or separated zone cells have been conducted in 14 other studies. Cells were established in culture as in above and then exposed to test reagents for 4-6 days, and the media were recovered daily for steroid human assays. In three of these studies, the effect of added low-density lipoprotein (LDL) has been addressed. We have not completed analysis of steroid levels in media from the above-mentioned studies.

We have many adrenal fragments that were either fixed in formalin, frozen at -70 C, or placed in cryopreservative and frozen. Fixed tissues can be analyzed for the presence of cytokines and their receptors utilizing appropriate immunohistochemistry reagents; tissues frozen will be used for homogenization and analysis by Western blot methods for steroidogenic enzymes, cytokines, and receptors and analysis by Northern blot methods for mRNA for the above; and, the cryofixed/frozen tissues will be used for analysis of cytokine binding by use of radiolabeled ligands and for in situ hybridization studies.

SIGNIFICANCE: We are developing promising data concerning in vivo and in vitro responses of the adrenal to illness and cytokines, respectively. Most notably, adrenal androgen production is impaired whereas cortisol production is increased in vivo with stress and trauma. We seek to replicate such findings in vitro in response to one or a combination of cytokines.

WORK PLAN (next 12 months): We will complete analysis of the effects of TGF- $\beta$  on adrenal steroid production in vitro. Studies of TGF $\beta$  and IL-1 $\beta$  on the adrenal will be replicated in the absence of serum to determine whether serum components may modify the steroidogenic or growth responses. We will study the effects of TGF $\alpha$  on adrenal growth and steroidogenesis in vitro. Since the Co-Investigator has perfected his assay methods for blood cytokine analysis as well as for Northern blot analysis of cytokine mRNA in blood lymphocytes, we will study the relationship between blood cytokines and adrenal steroidogenesis in healthy and stressed adults.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Harlin, C.A. and C.R. Parker, Jr. "Investigation of the effect of interleukin-1 $\beta$  on steroidogenesis in the human fetal adrenal gland". Steroids 56:72-76, 1991.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1453

R&T CODE: 4414833

PRINCIPAL INVESTIGATOR: Merrily Poth, M.D.

INSTITUTION: Uniformed Services University of the Health Sciences

GRANT TITLE: Interactions of Cachectin (Tumor Necrosis Factor) with Endocrine Systems

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 June 1990 - 31 Dec 1992

OBJECTIVE: To investigate the mechanisms of tumor necrosis factor activity in endocrine systems and conversely to study the effect of hormones on TNF production.

ACCOMPLISHMENTS (last 12 months): Our specific accomplishments in this period include:

1. Effect of TNF on adrenal function - We had previously reported that TNF significantly inhibited ACTH - stimulated glucocorticoid secretion. In studies to define mechanisms of this effect we found that TNF, even at the highest concentrations used, did not effect cell viability. We also examined the effect of preincubation with TNF prior to stimulation with ACTH. In these studies cells preincubated with TNF had normal responses to ACTH and no change in TNF-mediated inhibition of glucocorticoid secretion. Thus it appears that it is necessary for TNF to be present at the time of stimulation for inhibition to occur. In addition no acute down-regulation to TNF effect was seen with preincubation.

The stimulation of glucocorticoid release by ACTH is mediated by cAMP and dibutyryl cAMP stimulates the release of glucocorticoid in the adrenal cell preparation. This stimulation was also effectively inhibited by TNF. Based on this data it appears that TNF inhibition occurs distal to cAMP production. Neither phorbol ester nor prostaglandin synthesis inhibitor (indomethacin) had any effect on baseline or stimulated corticosterone secretion, nor on TNF inhibition of secretion.

These data are being prepared for publication at the present time.

2. Effect of TNF on Thyroid - We have shown that TNF, at concentrations commonly seen in serum of acutely ill patients, effectively blocks TSH stimulated function of human thyroid cells in culture. Again, as in adrenal cell culture, it does this without affecting cAMP production, implicating a locus of

action beyond adenyl cyclase. We also measured cGMP and found no effect of TNF on this system in thyroid cells. Neither incorporation of radioactive nucleotide nor cell viability was effected by TNF with or without TSH. These data are in press.

3. Hormonal control of TNF production - Although we initially found marked stimulation of TNF production by macrophages obtained from DHEA injected mice, on multiple attempts to repeat these experiments we were unable to duplicate our earlier positive results. We also found that mice injected with DHEA have increased sensitivity to LPS and that they had a persistent increase in serum TNF after a non-lethal dose of LPS. We are repeating these experiments and will publish data to resolve these discrepancies between the in vitro and in vivo systems.

SIGNIFICANCE: Tumor necrosis factor clearly has marked inhibitory effects on adrenal and thyroid function. This inhibition is seen at concentrations of TNF seen in the serum of patients with a wide variety of illnesses, including sepsis, trauma and HIV disease. Thus there is a possible role for TNF in the mediation of the perplexing abnormalities of thyroid function found in severe illness, the so-called "sick-euthyroid syndrome." The inhibition of ACTH-stimulated glucocorticoid secretion by TNF may have important therapeutic implications in the treatment of patients with sepsis, HIV disease and other conditions associated with TNF production.

WORK PLAN (next 12 months): We are planning to study the interrelationships of other cytokines with TNF in the function of adrenal and thyroid. We will also explore the mechanisms of TNF effects on adrenal and thyroid function. We are in the process of repeating the experiments showing increased TNF production in vivo in DHEA treated mice after LPS injection and will publish this data when experiments are completed.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Poth, M., Tseng, Y.L. and Wartofsky, L. (1991) Inhibition of TSH activation of human cultured thyroid cells by tumor necrosis factor: An explanation for decreased thyroid function in systemic illness? Thyroid (In Press).
2. Blauer, K.L., Poth, M., Rogers, W.M. and Burnten, E.W. Dehydroepiandrosterone antagonizes the suppressive effect of dexamethasone on lymphocyte proliferation. Submitted Endocrinology April 1991.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1095

R&T CODE: 4414004

PRINCIPAL INVESTIGATOR: Eric M. Smith, Ph.D.

INSTITUTION: University of Texas Medical Branch

GRANT TITLE: Molecular mechanisms for activation of the immune-neuroendocrine axis

REPORTING PERIOD: June 1, 1990 - May 31, 1991 (12 months)

AWARD PERIOD: November 1, 1990 - October 31, 1992

OBJECTIVE: This project narrowed its focus considerably when renewed November 1, 1990 and therefore two objectives are covered by this report. The initial objective was to determine if the hypothalamus can modulate immune responses directly by hypothalamic hormones, or indirectly through activation of other tissues to release cytokines. The renewal examines the mechanisms that activate the hypothalamic-pituitary-adrenal axis during virus infections. Specifically, we are looking at viral components or related synthetic compounds for an ability to induce lymphocyte production of neuroendocrine hormones either directly or through indirect mechanisms such as cytokines.

ACCOMPLISHMENTS: Our efforts in the past have centered largely around studying corticotropin (ACTH) production by lymphoid cells, particularly in regards to its induction by corticotropin releasing hormone (CRH) and thyrotropin releasing hormone (2) and endotoxin (1,3,6). In a collaboration with Dr. George B. Stefano (S.U.N.Y. College at Old Westbury) we have started using a new technology of digital video analysis that is a very sensitive measure of cellular activation. We found that ACTH would inactivate spontaneously activated human granulocytes and have tentatively identified its mechanism of action (7). Both ACTH and melanotropin (MSH) caused significant conformational changes plus a reduction in the locomotory activity of spontaneously active granulocytes. ACTH required 3h for maximal inactivation whereas MSH only required 30 min and was more potent than ACTH. The addition of phosphoramidon, a specific inhibitor of neutral endopeptidase 24.11 (also known as CD10), blocked ACTH inactivation of granulocytes. RIA analysis for MSH of supernatant fluids from granulocytes incubated with ACTH demonstrated a time dependent increase in the levels of MSH. In addition, phosphoramidon enhanced MSH inactivation of granulocyte cultures. These data strongly indicate that a large component of ACTH's activity is due to its processing to MSH by granulocyte associated NEP. Parallel experiments with invertebrate immunocytes from the mollusc Mytilus edulis gave similar results indicating the universality of this phenomenon. Also found, was that the human immunodeficiency virus, induces ACTH and MSH production in H9 T-lymphoma cells.

Our studies looking at specific viral components or synthetic substitutes have focused on the synthetic double stranded RNA,

polyribonucleosinic:polycytidylic acid. As predicted and similar to endotoxin it will induce mouse splenocytes to synthesize ACTH, detectable by RIA. We are now investigating the role of interferon (IFN)  $\alpha/\beta$  which is induced in this system concomitantly with ACTH.

SIGNIFICANCE: Considering our earlier work with Newcastle disease virus and bacterial lipopolysaccharide induction of ACTH, these results may suggest an important common role for this mechanism in immunosuppression. Rather than ACTH exerting the major immunomodulating effect, MSH is the mediator. Furthermore partial regulation may be involved at the cellular level, dependent upon the cell type, presence and levels of neutral endopeptidase. The results with Pi:C suggest that actual infection of the cells is not necessary, that viral products, in particular double stranded RNA might activate the neuroendocrine system.

WORKPLAN (next 12 months): The specific objective of next year's work is to investigate the role of cytokines (IL-1,-2,-6, TNF, and IFN  $\alpha/\beta$ ) in the induction of ACTH, MSH, and endorphins. We will examine these for induction and feedback inhibition of one and another. Other viral subcomponents will be examined for activity, including viral double stranded RNA, inactivated influenza vaccine and viral proteins.

PUBLICATIONS:(reviews and abstracts not listed due to space)

1. Hughes, T.K., E.M. Smith, J.A. Barnett, R. Charles, and G.B. Stefano. (1991) LPS stimulation of invertebrate hemocytes: A role for immunoreactive TNF and IL-1. Develop. Comp. Immunol. 15: 117-122.
2. Harbour, D.V., T.E. Kruger, E.M. Smith, and W.J. Meyer, III. 1991. Detection and partial characterization of thyrotropin-releasing hormone (TRH) receptors on a T-cell line. Neuroendocrinology (in press).
3. Harbour, D.V., F.S. Galin, T.K. Hughes, E.M. Smith and J.E. Blalock. 1991. Role of the leukocyte-derived POMC peptides in endotoxic shock. Trends in Shock Research (in press).
4. Stefano, G.B., E.M. Smith, and T.K. Hughes. 1991. Opioid induction of immunoreactive interleukin-1 in Mytilus edulis and human immunocytes: An interleukin-1-like substance in invertebrate neural tissue. J. Neuroimmunol. (In press).
5. Stefano, G.B., D. Kimura, P. Finn, M.K. Leung, M.A. Shipp, E.M. Smith and T.K. Hughes. 1991. Immunoreactive met-enkephalin levels and the percent of opioid responsive cells in circulation in vertebrates and invertebrates change with age. Prog. NeuroEndocrinImmunol. (In press)
6. Hughes, T.K., E.M. Smith, J.A. Barnett, R. Charles, M.K. Leung and G.B. Stefano. 1991. Activation of distinct populations of Mytilus edulis immunocytes by endotoxin and opioids. Cell Tissue Res. (In press)
7. Smith, E.M., T.K. Hughes, F. Hashemi and G.B. Stefano. 1991. Conversion of ACTH to MSH in immune modulation: Implications for the human immunodeficiency virus. (Submitted to P.N.A.S.)

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1962

R&T CODE: 4414935

PRINCIPAL INVESTIGATORS: Drs. G.J. Stanton and T.K. Hughes

INSTITUTION: University of Texas Medical Branch

GRANT TITLE: Systemic Activation of the IFN System by Exposure of Natural Epithelia to Physiologic (Low Dose) Levels of IFN

REPORTING PERIOD: 1 July 1990 - 30 June 1991 (12 months)

AWARD PERIOD: 1 July 1989 - 30 June 1991

OBJECTIVE: To determine the mechanisms involved in systemic modulation of the antiviral, immune-, and hormonal systems initiated by exposure of mucosal surfaces to low concentrations of IFN.

ACCOMPLISHMENTS: (last 12 months): We had previously found that syngeneic lymphocytes treated with various concentrations of IFN and injected IP or intranasally into mice could protect them from lethal Semliki Forest virus (SFV) infection. The protection was biphasic since lymphocytes treated with low (0 to 30 IU/ml) concentrations of IFN were more protective than those treated with higher (>50). These findings indicated that a unique, ie. biphasic protective system could be systemically modulated by a process initiated by IFN treated cells. Since then numerous studies have been performed in mice to determine whether the system also could be activated by applying IFN directly to mucosal surfaces. In these studies we chose to give the IFN concentrations in drinking water maintained at pH 2 since A) the IFN was very stable at this pH, B) bacterial contamination was eliminated, C) the mice drank adequate amounts to bathe the mucosa (~5 ml/day), and D) excessive handling or anesthetization of mice was not required. We found that a biphasic protective response against SFV occurred in 10 out of 13 experiments. Two of the 13 experiments that did not show a biphasic response did not contain a group of mice that received a sufficiently high concentration of IFN (>100 IU/ml) to expect this response to occur. One group responded minimally to all concentrations of IFN used. The ANOVA P values for the significance of a protective effect occurring between mock IFN treated groups and groups receiving 1.0 to 300 IU/ml was <.0005, and loss of a protective effect between groups receiving 1 to 300 IU/ml and 1000/ml was .04. Thus the protection was significant and biphasic. Other important observations were that; A) The length of time for applying the IFN could be reduced from 6 days preinfection and 10 days postinfection (P.I.) to 2 days pre- and 2 days P.I.; thus the development of protection was rapid, B) treatment had to continue 2 days P.I. although there was 50 to 100,000 units of IFN/ml in the blood of infected mice at that time that did not protect; thus a different IFN system was most likely activated by the oral administration; and C) recombinant IFN gave the same results as natural

IFN and the protective effects could be abolished by inactivating the IFN; thus the protective effects were most likely due to the IFN present in the preparations.

SIGNIFICANCE: The studies indicated that a unique biphasic system of protection could be activated by oral application of low concentrations of IFN. Because the effective concentrations of IFN were very low suggested the protection was most likely not due to the direct effects of IFN getting into the blood and that a considerable amount of amplification had to occur from interactions starting at the local sites of application. One possibility that could explain these findings could be that IFN interacts with highly innervated tissues in the upper G.I. and respiratory tracts and somehow communicates messages to the CNS causing release of neural peptides that are antiviral and/or modulate host resistant systems including the immune system. Preliminary studies support this hypothesis. The ability to modulate these systems has obvious implications.

WORK PLAN: We plan to investigate the nature and significance of the above mentioned mechanism on the hormonal, antiviral, antibody plaque forming response to SRBC, NK, and/or mitogenic activity to lectins in mice by: A) using locally or systemically applied nerve blocking agents to inhibit initiation or amplification of systems eg. lanthanum chloride, actinomycin D, naloxone, B) determination of treatment effects on hormonal fluctuations in serum of mice, C) deletion experiments using antibodies to hormones, IFNs and other cytokines that mediate and amplify immune effects.

#### PUBLICATIONS AND ABSTRACTS:

1. Fleischmann, W.R., Fields, E.E., Wang, J.L., Hughes, T.K., and Stanton G.J. (1991) Modulation of peripheral leukocyte counts in mice by oral administration of interferons. Proc. Soc. Exp. Biol. Med., in press
2. Stanton, G.J., Hughes T.K., Heard, H.H., Georgiades, J. and Whorton, E. (1990) Modulation of a natural virus defense system by low concentrations of IFN at mucosal surfaces. Abstract presented at Annual meeting of the International Society for IFN Research, San Francisco, Ca, Nov. 14-18.

CELL BIOLOGY OF TRAUMA  
ACCELERATED RESEARCH INITIATIVE

SCIENTIFIC OFFICER: Dr. Constance Oliver

BEGAN: OCTOBER 1, 1989

ENDS: SEPTEMBER 30, 1993

PROGRAM OBJECTIVE: TO DEVELOP IN VITRO MODELS FOR INVESTIGATION OF THE EFFECTS OF HYPOXIA ON CELLULAR SIGNAL TRANSDUCTION AND ION REGULATION.

NAVY OBJECTIVE: TO DEVELOP NEW DIRECTIONS FOR DESIGN OF THERAPEUTICS FOR COMBAT CASUALTY CARE.



## ANNUAL PROGRESS REPORT

Grant #: N00014-88-K-0395

R & T CODE: 441q805

PRINCIPAL INVESTIGATOR: Margaret S. Burns, Ph.D.

INSTITUTION: University of California, Davis

GRANT TITLE: Intracellular Signalling in Retinal Ischemia

REPORTING PERIOD: 1 July 1990 - 31 May 1991 (12 months)

AWARD PERIOD: 1 July 1988 - 30 June 1991

OBJECTIVE: To determine if Central Nervous System (CNS; retinal) ischemia is characterized by increases of total calcium and in which cell layers this occurs; to determine if this occurs by influx of calcium or redistribution of endogenous calcium; to determine what accompanying changes there are in other cellular electrolytes; to determine if these changes are similar *in vivo* and *in vitro*; to determine if changes in intracellular energy stores and signalling mechanisms are altered in ischemia.

ACCOMPLISHMENTS (last 12 months): Using an explant system of retinal tissue incubated in modified Ames' medium (an artificial cerebrospinal fluid), we showed the following in preliminary experiments:

- 1) Secondary Ion Mass Spectrometry (SIMS) has the technical capacity to answer biological questions discriminating electrolyte exchange from accumulation.
- 2) Total calcium was not increased in retina incubated under either oxygen rich or presumably anoxic (see below) conditions.
- 3) Total calcium was not increased in retina incubated with high glutamate concentrations, a conditions presumed to mimic CNS ischemia.
- 4) The rate of exchange of endogenous calcium with exogenous calcium was very rapid - 75% replacement within 30 minutes.
- 5) There was no particular retinal cell layer that was appreciably different from another.
- 6) There was an increase in radioactive calcium-45 in the retina under the incubation conditions.
- 7) There appeared to be loss of Mg from the retina during the incubation conditions.

As was apparent at the ONR Workshop for this program, the levels of oxygen in tissue cannot be assumed to be zero when tissue medium is gassed with 100% nitrogen, we consider the above data to be preliminary, and have taken technical steps to be able to directly monitor oxygen concentrations during the incubations. Using a Stoelting brain slice monitor and Clark oxygen electrodes, we now have a set up to accurately monitor medium oxygen concentrations in the

low range. We have also adapted several assay systems to be able to monitor cell death, and cell lysis in a more quantitative manner, in order to more sensitively determine what is occurring in the tissue during ischemic perfusion.

**SIGNIFICANCE:** These preliminary results, using a novel technique for biological analysis, Secondary Ion Mass Spectrometry (SIMS), have the capacity to discriminate between tissue exchange of calcium and accumulation, that is not possible to do with other currently existing techniques. This could make possible a more detailed analysis of calcium homeostasis than is currently done.

**WORK PLAN (next 12 months):** Since SIMS is a relatively new technique to the biological community, it is necessary to proceed with experiments that can compare this novel measurement with other, more accepted measurements, of tissue electrolytes. Therefore, we will repeat the above experiments (and some variations) with concomitant measures of total tissue electrolytes by atomic absorption spectrophotometry. We will also look more closely at radioactive 45-Ca uptake in tissue and correlate it with the SIMS measurements to discriminate "accumulation" from exchange of electrolytes. It is possible to do this also with potassium, using both the radioactive substitute tracer 86-Rb, and the stable isotope 85-Rb. Based on what we have learned in these first experiments, we will concentrate on the initial phase of electrolyte exchange, during the first 30 minutes of incubation, in addition to the changes occurring at longer time periods, such as 2 to 6 hours (in order to compare reperfusion experiments at the longer time periods).

The in vitro experiments will be done with normoxia, and calibrated anoxia and perhaps hypoxia, in order to determine how these conditions affect electrolyte exchange. Also, since the presence of glutamate appeared to show an anomalous effect on calcium exchange in the photoreceptor cell, we will pursue these experiments to determine if this effect is real. The possible role of magnesium in ischemia induced damage will also be studied.

We plan, then, to proceed to in vivo experiments, using a model of ligation of the central retinal artery in rats, to determine if similar electrolyte changes occur as in vitro. A series of perfusion, reperfusion experiments will be done to see if this CNS tissue (retina) can be rescued from ischemic damage.

**PUBLICATIONS AND ABSTRACTS (last 12 months):**

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2. Burns, M.S. and Tyler, N.K. (1990) Selective neovascularization of the retinal pigment epithelium in rat photoreceptor degeneration in vivo. Curr Eye Res 9:1061-1075.
3. Burns, M.S. and Panattoni, C.M. (1990) Calcium localization in retina in glutamate toxicity and ischemia in vitro. Abstract. Soc Neurosci. Neurosci 16:190.
4. Burns, M.S., Panattoni, C.M., Hitzman, C. and Lux, G. (1991) Stable isotopic analysis of ion movement in retina. Abstract. 8th Int'l Conf on SIMS. Amsterdam, Sept. 1991.

# ANNUAL PROGRESS REPORT

GRANT #: N00014-88-J-1137

R&T CODE: 441q803

PRINCIPAL INVESTIGATOR: Robert J. Cohen

INSTITUTION: University of Florida

GRANT TITLE: The Effect of Ischemia on Cellular Levels of Cyclic Nucleotides in a Cone-Dominant Retina

REPORT PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 15 June 1988 - 30 September 1991

OBJECTIVE: To examine the effect of ischemia on the levels and light modulation of cyclic nucleotides and energy metabolites in the cone-dominant retinas of sighted and blind chickens.

ACCOMPLISHMENTS: We examined the effects of five minutes and twenty minutes of ischemia on ATP levels and cyclic nucleotide levels in whole retinal complexes in light and dark adapted sighted and blind chickens. ATP levels were decreased substantially in all four groups. The decrease is less than reported for rod dominant retina systems. We determined that after five minutes of ischemia, levels of cyclic AMP and cyclic GMP were not different for ischemic and nonischemic retina of sighted birds, both light- and dark-adapted. For the nontransducing rd retina, levels of cyclic GMP increased fivefold from an abnormally low level to a level comparable to the level in the retina of the sighted chicken. The effect of ischemia on cyclic GMP levels in the rd retina is unlikely to be due to the decreased levels of ATP since the effect is not observed in the sighted chick. Examination of levels at twenty minutes is complicated by the fact that the retina tissue is fragile and perhaps damaged by the prolonged ischemic condition.

SIGNIFICANCE: The chicken cone-dominant retina contrary to expectations is less sensitive to ischemia than the rod dominant-retina. The rd model has proved its value in suggesting that the results of ischemia at the molecular level may not be mediated through changes in the level of ATP.

WORK PLAN: We will continue to work on the retina at twenty minutes of ischemia. The levels of ATP, creative phosphate, cyclic AMP and cyclic GMP will be examined in light adapted and dark adapted tissue. Depending on the results, either ten minutes of ischemia or less likely thirty minutes of ischemia will be examined. Microdissection of retina layers and microanalysis of cyclic nucleotides and ATP will be performed.

INVENTIONS: None.

PUBLICATIONS AND ABSTRACTS (last twelve months): Ph.D. Dissertation, Cyclic Nucleotides in the (rd) retinal degenerate chicken retina, Nancy R. Lee, 31 July 1991.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1738

R&T CODE: 441q400

PRINCIPAL INVESTIGATOR: Thomas M. Devlin, Ph.D.

INSTITUTION: Hahnemann University School of Medicine

GRANT TITLE: Studies of Control of Cytosolic Calcium Concentration in Stress and Tissue Ischemia: Role of Calciphor (Oligomers of Prostaglandin B<sub>1</sub>)

REPORTING PERIOD: 1 June 1990 - 30 May 1991

AWARD PERIOD: 1 March 1989 - 30 June 1991

OBJECTIVE: To determine the mode of action of di- and tri-Calciphor (dimer and trimer of 16,16'-dimethylprostaglandin B<sub>1</sub>) in controlling cellular Ca<sup>2+</sup> and to evaluate the effect of Calciphor in cerebral ischemia in gerbils.

ACCOMPLISHMENTS: In normal gerbils Calciphor (10 mg/kg, i.p.) reduced locomotor activity by about 50% in 2 hrs; normal activity returned in 5 hrs. Average voltages and high frequency discharges in the EEG were reduced. Di- and tri-Calciphor improved equally the survival of gerbils subjected to cerebral ischemia when administered (10 mg/kg) at 5 min plus 24 hrs postischemia; 14 day survival: controls, 36%, di-Calciphor, 68%, and tri-Calciphor, 64%. The same protection occurred when administered at 3 hrs plus 24 hrs postischemia. Calciphor reduced the EEG hyperactivity and seizures. Postischemic treatment with di-Calciphor reduced postischemic neuropathology; > 90% of the hippocampus neurons were preserved at 6 days postischemia. With no treatment few intact neurons and pronounced gliosis affected the CA1, 2 and 3 sectors. Calciphor prevented proteolysis, presumably by the Ca<sup>2+</sup> dependent protease, calpain I, of spectrin and MAP2 in the forebrain.

Preloading isolated rat liver mitochondria with fluo-3, a Ca<sup>2+</sup> fluorescent indicator, permits monitoring of matrix free Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>m</sub>). [Ca<sup>2+</sup>]<sub>m</sub> is not directly controlled by cytosolic Ca<sup>2+</sup>; Mg<sup>2+</sup>, phosphate, ATP and ADP concentrations influence [Ca<sup>2+</sup>]<sub>m</sub>. Calciphor reduced the [Ca<sup>2+</sup>]<sub>m</sub> under all conditions tested; the mechanism appears to involve a shift from [Ca<sup>2+</sup>]<sub>m</sub> to sequestered (bound) Ca<sup>2+</sup>. Attempts to correlate changes in [Ca<sup>2+</sup>]<sub>m</sub> with matrix pH have not been successful. [Ca<sup>2+</sup>]<sub>m</sub> at 300 nM and higher inhibits citrulline synthesis; di-Calciphor stimulated citrulline synthesis at high [Ca<sup>2+</sup>]<sub>m</sub> by lowering [Ca<sup>2+</sup>]<sub>m</sub>. Di-Calciphor inhibited citrulline synthesis at [Ca<sup>2+</sup>]<sub>m</sub> of 100 nM (optimal for citrulline synthesis); the [Ca<sup>2+</sup>]<sub>m</sub> was reduced to less than 50 nM. [Ca<sup>2+</sup>]<sub>m</sub> may have a biphasic effect on citrulline synthesis or perhaps di-Calciphor may affect the concentration of another divalent cation.

Di- and tri-Calciphor reduced extra-mitochondrial steady-state Ca<sup>2+</sup> levels (800 nM) to 100-200 nM. The increase in the Ca<sup>2+</sup> buffering capacity of mitochondria could lead to a significant role in maintaining cytosolic Ca<sup>2+</sup> levels.

WORK PLAN: Gerbil ischemia model: Evaluation of times of injection of Calciphor on gerbil survival during the first 3 days. MK-801, which protects in regional cerebral ischemia,

will be tested in the gerbil model. A comprehensive postischemic evaluation, including blood pressure, cardiac rate, EEG recover, postischemic seizures and mobility, will be conducted. Histopathology studies will be completed.

**Mitochondrial Matrix  $\text{Ca}^{2+}$ :** Determine if the effect of Calciphor on  $[\text{Ca}^{2+}]_m$  is due to changes in mitochondrial matrix pH. It is possible that the primary effect is to induce a pH change which could lead to changes in ionization of  $\text{Ca}^{2+}$  binding sites. Other conditions (redox state, phosphate potential, anoxia, etc.) will be evaluated. Studies with isolated gerbil brain mitochondria will be conducted to correlate the in vitro and in vivo studies.

Calciphor inhibits lipid peroxidation in isolated mitochondria (reported in 1989-90); the mechanism of inhibition and the effect on lipid peroxidation of other membrane systems will be evaluated.

**SIGNIFICANCE:** Calciphor should be an effective therapeutic agent in humans to protect against the tissue damage following an ischemic episode (e.g. stroke, myocardial infarct, shock, organ transplant, etc.). The drug, when administered following the ischemic period, prevents loss of mitochondrial function in situ in rat kidneys, and improves survival and brain function of gerbils subjected to total cerebral ischemia. The mode of action of Calciphor may be to protect mitochondrial function which is damaged during anoxia and tissue reperfusion. The drug has several effects on mitochondrial function, including protection of energy transduction, maintenance of integrity, and reduction of  $[\text{Ca}^{2+}]_m$ , which are unique activities, suggesting that Calciphor is a new class of drugs, with totally new pharmacological properties, for treating tissue ischemia.

#### PUBLICATIONS AND ABSTRACTS

1. Saavedra-Molina, A. and Devlin, T.M. (1991) Effect of extra- and intramitochondrial free calcium on citrulline synthesis. FASEB J. 5, A1182. (poster presentation)
2. von Lubitz, D.K.J.E., Devlin, T.M., Kalenak, A., Lin, R.C.S., Matesic, D.F. and McKenzie, R.J. (1991) Cerebral ischemia in gerbils: Therapeutic effects of delayed treatment with Calciphor (dimer of 16,16'dimethylprostaglandin  $\text{B}_1$ ). Abst. submitted for meeting of Soc. for Neuroscience, 1991.
3. Matesic, D.F., Devlin, T.M., Kalenak, A., McKenzie, R.J., von Lubitz, D.K.J.E. and Lin, R.C.S. (1991) Ischemia in gerbil forebrain: Postischemic treatment of di-Calciphor prevents calcium mediated degradation of spectrum and MAP-2. Abst. submitted for meeting of Soc. for Neuroscience, 1991.
4. McKenzie, R., von Lubitz, D.K.J.E., Kalenak, A., Lin, R.C.S. and Devlin, T.M. (1991) Depressant effects of Calciphor (dimer of 16,16'dimethyl prostaglandin  $\text{B}_1$ ) on EEG, locomotor activity and muscular coordination in mongolian gerbils. Submitted for publication to Neuroscience Letters.
5. von Lubitz, D.K.J.E., Kalenak, A., McKenzie, R.J., Lin, R.C.S. and Devlin, T.M. (1991) Treatment of severe forebrain ischemia in gerbils: the effect of di- and tri-Calciphor (dimer and trimer of 16,16'dimethyl prostaglandin  $\text{B}_1$ ). Submitted for publication to J. Exp. Pharm. and Therapeutics.

ANNUAL PROGRESS REPORT

GP'NT # N00014-89-J-3051

R&T CODE: 400x065yip04

PRINCIPAL INVESTIGATOR: Jordan B. Fishman, Ph.D.

INSTITUTION: University of Massachusetts Medical School

GRANT TITLE: Modulation of Neuronal Signal Transduction by Gangliosides

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 July 1989 - 30 June 1992

OBJECTIVE: To investigate the role of gangliosides in signal transduction in the liver and brain in an attempt to elucidate the role gangliosides play in receptor function, receptor/G-protein interaction, and regulation of cell function.

ACCOMPLISHMENTS (last 12 months): Two publications, outlining work on ganglioside changes during liver regeneration and on the potential of G-proteins as molecular targets for neurotoxins have been published. We are finishing the work on the effect of gangliosides on vasopressin V1 receptor function, which we hope to submit to a journal this summer. In addition, we have continued with our work on the potential cytoprotective role of gangliosides following exposure to organic cytotoxins such as carbon tetrachloride. It appears that gangliosides can, indeed, help protect the liver from membrane decomposition under some circumstances, but the expense and time required for a full scale analysis of this phenomenon may require the submission of a new contract to cover this work. We are in the process of expressing the vasopressin V1 receptor in mammalian cells, which should allow us to study the effect of gangliosides on the signal transduction pathway, which will complement well the previous work (see above) in which the effect of gangliosides on V1 receptor binding was determined.

SIGNIFICANCE: Three major significant findings have emerged from the work of the past 12 months: (1) that neurotoxins may act in a specific manner, targeting protein(s) involved in the transduction of receptor-dependent signals, which may lead to impairment of intraneuronal communication; (2) gangliosides play an important role in the regulation of receptor function, including modulation of hormone-dependent cell growth and receptor kinetics; and (3) upon exposure to chemicals such as carbon tetrachloride, a hepatotoxin, gangliosides appear to have the ability to decrease cell surface blebbing and increase cell viability, suggesting they may have a role in cytoprotection in the field in the event of the use of such agents or agents which work in similar fashion.

WORK PLAN (next 12 months): As described above, in order to more fully understand the role of gangliosides in receptor function, we feel it is necessary to express the V1 receptor in a cell line which does not have vasopressin V1 receptors expressed endogenously. This will allow us to add not only gangliosides and receptor at the levels we wish, but in addition, we will be able to modify the receptor gene in such a way as to identify the site(s) of interaction between the receptor and gangliosides. This would be hard to do in a cell line which was already expressing V1 receptors. We also hope to isolate the gene for the V1 receptor using expression in COS-7 cells and ligand binding to detect expressed receptors. We have recently expressed the V1 receptor in COS-7 cells, and hope to have the receptor clone isolated in the near future. This latter work is supported by the NIH, but the isolation of the clone will allow us to further our ONR-supported research interests.

#### PUBLICATIONS AND ABSTRACTS:

1. Fishman, J.B., Cahill, M., Morin, P., McCrory, M., Bucher, N.L.R., and Ullman, M.D. (1991) Specific Gangliosides Increase Rapidly in Rat Liver Following Partial Hepatectomy. *Biochem. Biophys. Res. Commun.* 174, 638-646.
2. Fishman, J.B., Rubins, J.B., Chen, J.-C., Dickey, B.F., and Volicer, L. (1991) Modification of Brain Guanine Nucleotide-Binding Regulatory Proteins by Tryptamine-4,5-Dione, a Neurotoxic Derivative of Serotonin. *J. Neurochem.* 56, 1851-1854.
3. Fishman, J.B. and Dickey, B.F. (1990) The Vasopressin V1 Receptor: Purification, Characterization and Analysis of V1 Receptor Interaction(s) with Guanine Nucleotide-Binding Proteins. In: Receptor Purification (G. Litwack, ed.), Humana Press, pp. 337-353.
4. Chen, J.-C., Clancy, J.F., Triestman, S.N., and Fishman, J.B. (1990) Expression of Liver and Hippocampal Vasopressin V1 Receptors in Xenopus Oocytes by Microinjection of Poly(A)<sup>+</sup>RNA. *J. Cell Biol.* 111, 336a.



## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1898

R&T CODE: 441q013

PRINCIPAL INVESTIGATOR: Gordon A. Jamieson, Jr., Ph.D.

INSTITUTION: University of Cincinnati College of Medicine

GRANT TITLE: Molecular Mechanisms Regulating Human Fibroblast Growth

REPORTING PERIOD: 1 June 1990 - 31 May 1991 (12 months)

AWARD PERIOD: 1 June 1990 - 31 May 1992

OBJECTIVE: To identify and characterize cDNAs for novel members of the steroid/hormone receptor superfamily whose function is eicosanoid-dependent.

ACCOMPLISHMENTS (past 12 months): We reduced the scope of our research project since funding was provided for two of the three years requested. We decided to focus on the most interesting portion of our original research proposal; the identification of eicosanoid-dependent transcriptional factors. Our revised approach is molecular in nature and takes advantage of known structural and functional aspects of the steroid/hormone receptor superfamily. Of particular relevance to our study are the Zn-finger (located in the central portion of these molecules) and the ligand binding domains (located COOH terminal from the Zn-finger domain) of these proteins. The Zn-finger region has been determined to function as the site of sequence-specific interaction with DNA and is a relatively highly conserved structure. Conversely the ligand binding domains of different members of the steroid/hormone receptor superfamily possess only limited structural similarity. We have synthesized degenerate oligonucleotides primers, based upon the DNA sequence of the Zn-finger domains of several members of the steroid/hormone superfamily, for use in anchored polymerase chain reactions (PCR). This approach is designed to provide for the amplification of regions of members of this receptor family 3' to the Zn-finger:DNA-binding domain - i.e. the region corresponding to the ligand binding domain. RNA and/or mRNA was isolated from human fibroblast cultures, reverse-transcribed and anchored PCR amplifications performed under a variety of conditions. It has proven difficult to obtain conditions which allow the efficient amplification of the 3'-portion of the steroid/hormone receptors despite our ability to readily amplify portions of a control gene (B-actin) using anchored PCR. Optimization of our experimental conditions continues using our positive control, the glucocorticoid/dexamethasone receptor. We surmise that our procedural difficulties result from the large 3' untranslated region present in many members of this gene family. Hence efficient amplification reactions are difficult to perform. In an attempt to develop an alternate procedure we have begun to use our Zn-finger based degenerate primers to prime second strand cDNA synthesis and then, subsequent to a series of modifying reactions, attempt to insert the material obtained into a fusion protein expression vector (see below). These studies are ongoing.

Our next step will be to insert our PCR amplified material [containing the putative ligand binding region(s)] into a bacterial fusion protein expression vector. This will allow us to express the ligand binding portion of

these molecules as part of a bacterial fusion protein. The NH<sub>2</sub>-terminal portion of this fusion protein (maltose binding protein) allows for the rapid affinity purification of microgram quantities of the fusion protein which we will use in ligand binding assays. (Addition of a random number of dG residues and flushing of the ends with DNA polymerase will be required prior to insertion into this vector to allow for various potential reading frames.) To verify our ability to successfully insert sequences into a fusion protein expression vector in the correct reading frame we are using the region encoding the ligand binding region of the human glucocorticoid receptor. Affinity purification and ligand binding procedures will be standardized using this maltose binding dexamethasone fusion protein construct. These studies are in progress.

After we overcome the procedural difficulties noted above, these experiments should result in the production of large quantities of fusion protein for use in ligand screening assays and partial cDNAs for use in DNA sequence analyses, Northern analyses of gene expression and the probing of cDNA libraries for full-length cDNA clones for these proteins. Our revised strategy has the advantage that it should detect members of the steroid-hormone receptor superfamily even if they do not bind eicosanoids, which our original proposal would not have detected. The prime disadvantage of our current approach is its inability to detect molecules which do not contain significant homology to the DNA-binding regions of known steroid-hormone receptors. However in future studies this disadvantage can be remedied by using a different set of degenerate oligonucleotide PCR primers. Clearly the most difficult portion of this project is the successful completion of the amplification, modification and insertion of amplified material into the fusion protein expression vector so that we can commence radioligand binding assays.

**SIGNIFICANCE:** This project may allow us to identify molecules which regulate gene transcription in an eicosanoid dependent fashion. This would provide for the development of a variety of therapeutic regimens which may allow one to enhance cellular proliferative responses subsequent to traumatic injury.

**WORK PLAN (next 12 months):** During the final funding period we will continue the development and implementation of our PCR amplification, expression vector and radioligand binding protocols. Due to the difficult nature of the PCR portion of our cloning strategy we are working on these two aspects in parallel. Continued experimentation should provide us with cloned ligand binding regions and an assay for use in characterizing their ligand binding properties. Concurrently we will also insert the ligand binding region of N10 into our fusion protein expression system - so that we can test its ligand binding properties. N10 is of particular interest since its expression is induced by serum. Hence it will be of interest to examine the ability of serum and different growth factors to stimulate the expression of known immediate early genes (*fos*, *jun*, *EGR1/2*) versus the level of induction different members of the steroid/hormone receptor superfamily [retinoic acid (RAR, RXR), glucocorticoids (DEX) and N10]. Time permitting we will evaluate effects of a variety of eicosanoids on cell growth (i.e. stimulation of [<sup>3</sup>H]-thymidine incorporation), and the ability of cultured human fibroblasts to metabolize arachidonic and linoleic acids into a variety of eicosanoids.

# ANNUAL PROGRESS REPORT

Grant #: N00014-89-J-1098

R&T CODE 441q806

PRINCIPAL INVESTIGATOR: Marvin A. Karasek, Ph.D.

INSTITUTION: Stanford University

GRANT TITLE: Role of Second Messengers in Ischemic Tissue Damage

REPORTING PERIOD: 31 May 1990 - 1 June 1991

AWARD PERIOD: 1 November 1989 - 30 October 1991.

OBJECTIVE: To develop an in vitro model of ischemic injury of the microvascular system with which to characterize the morphological, biochemical, and molecular changes occurring following ischemic injury.

ACCOMPLISHMENTS: Loss of the integrity in the gap and tight junctions in microvascular endothelial cells following ischemia results in the major shifts in tissue and blood fluids observed following reperfusion. We have now characterized how the integrity of the microvasculature is maintained, and the changes in the second messengers that results in the major changes in the endothelial cell cytoskeleton resulting in the loss of homeostasis. When intracellular levels of cyclic AMP are elevated either by the addition of exogenous dibutyryl cyclic AMP or by the addition of agonists that raise intracellular levels of cyclic AMP, the normal epithelioid configuration of the endothelial cells is maintained. In contrast, when cyclic AMP levels are decreased and protein kinase C is activated by an increase in calcium or by the addition of phorbol myristate acetate, a dramatic change in structure takes place. Using digital fluorescence microscopy, we have demonstrated that these changes in calcium occur in the nucleus of the endothelial cell, and that this colocalizes with one isoform of PKC. This coordinated response of changes in calcium, protein kinase C and vimentin provides us with the first basic information on how the homeostatic functions of the microvasculature are maintained.

When exposed to low oxygen tensions (1-2%) for prolonged periods (up to 48 hours), no significant changes in structure can be detected at either the light or electron microscopic levels. These cell populations do not show major changes in the binding of neutrophils. However if the inflammatory mediatory IL-1 is added to the cells, a major increase in neutrophil binding occurs early suggesting that cytokines released following injury promote the damage caused by neutrophils in the hypoxic tissues. These changes duplicate the increased neutrophil adhesion that are observed to take place in vivo. Under more stringent ischemic conditions when the oxygen tension is lowered to 0.003% major changes then take place in both the morphology and the metabolism of the microvascular endothelial cell even in the absence of neutrophils. Labeling of endothelial cell populations under these conditions with [<sup>35</sup>S]-methionine and 2-dimensional gel

electrophoresis of the synthesized proteins demonstrated a failure of the microvascular endothelial cell to synthesize an 80K protein synthesized by other vascular and non-vascular cell types (keratinocytes, squamous cell carcinoma, fibroblasts and large blood vessel cells). The microvascular endothelial cell appears to be unique in the lack of induction of this protein by hypoxia.

SIGNIFICANCE: Our understanding of how changes in second messengers control the shape of endothelial cells, our demonstration that hypoxic conditions and cytokines can increase neutrophil binding and alter endothelial cell morphology, and our demonstration of the failure of endothelial cells to synthesize a protein stimulated by hypoxia in other cell types provides us with important clues in how to begin to pharmacologically alter the consequences of either short term or prolonged ischemia. It is highly likely that the major tissue damage produced by ischemia can be altered if these biochemical changes resulting in altered morphology and biochemistry can be inhibited.

WORK PLAN: The work plan for the next grant period will be to further identify the cytokines released during periods of hypoxia that result in morphologic changes and to determine how these cytokines affect both neutrophil and monocyte interaction with endothelial cells. The 80 K protein inhibited by hypoxia will be further characterized, and its ability to prevent the morphologic changes and damage produced by neutrophils will be determined.

#### PUBLICATIONS AND ABSTRACTS

1. Mansbridge J, Murphy B, Morhenn V and Karasek M. (1991) Mechanisms of hypoxic injury to endothelial cells. Clinical Res. 39:490A.
2. Karasek M, Lipton B and Mansbridge J. (1991) Induction of macrophage-specific markers in activated skin microvascular endothelial cells. Clinical Res. 39: 488A.
3. Lipton B and Karasek M. (1991) Factors controlling microvascular endothelial cell transdifferentiation. Clinical Res. 39: 490A.
4. Lipton B, Bensch K and Karasek M. (1991) Transdifferentiation of microvascular endothelial cells: phenotypic characterization. Differentiation 46: 117-133.
5. Lipton B and Karasek M. (1991) Coordinated response of  $Ca^{2+}$  protein kinase C and vimentin in histamine treated skin microvascular endothelial cells. Clin. Res. 39: 16A.
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## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1433

R&T CODE: 441q908

PRINCIPAL INVESTIGATOR: John J. Lemasters

INSTITUTION: University of North Carolina at Chapel Hill

GRANT TITLE: Rescue of Injured Myocytes

REPORTING PERIOD: 1 June 1990 to 31 May 1991

AWARD PERIOD: 1 December 1988 to 30 November 1991

**OBJECTIVE:** To characterize injury to single cardiac myocytes and other cells by multiparameter digitized video microscopy (MDVM) and to evaluate protective agents and stabilization regimes.

**ACCOMPLISHMENTS (last 12 months):** The crucial events leading to loss of cell viability after hypoxic and toxic injury remain poorly understood. Cell death is rarely synchronous, and rapidly occurring events leading to irreversible injury may be obscured when large cell populations are studied. To overcome this drawback, single cultured hepatocytes and myocytes during hypoxic and toxic stress were studied using a new, rapidly developing technology called Multiparameter Digitized Video Microscopy (MDVM). In MDVM, living cells are labeled with multiple fluorescent probes. Each probe responds to a defined cellular parameter by emitting fluorescence of a specific wavelength. 2-dimensional images of the fluorescence of each probe are digitized and stored during the course of cell injury. In this way, several cellular parameters are monitored quantitatively over time. Examples of parameters which we have observed with spatial and temporal resolution in individual living cells include cytosolic free  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{H}^{+}$  and  $\text{Na}^{+}$ , mitochondrial membrane potential, cell viability, lipid order (fluidity), and protein and non-protein thiols. Our major findings are: 1. Formation of plasma membrane blebs accompanies toxic and hypoxic injury. In hepatocytes, blebs initially are sites of increased membrane fluidity but later a transition to gel state occurs shortly before the onset of cell death. 2. Cell death happens abruptly and is preceded by a metastable period of increasing non-specific permeability of the plasma membrane. Loss of viability often appears precipitated by rupture of a surface bleb. 3. An increase of cytosolic free  $\text{Ca}^{2+}$  is not a final common pathway leading to blebbing and cell death during hypoxic and oxidative stress. 4. A decrease of mitochondrial membrane potential precedes loss of cell viability and occurs early in some types of oxidative injury. 5. Cytosolic pH falls by more than 1 pH unit in parallel with ATP depletion and does not rise until a few minutes prior to the onset of cell death (metastable state). 6. Intracellular acidosis protects against cell killing during anoxic and toxic cell injury. Moreover, restoring acidotic pH to physiological levels greatly accelerates cell killing. This 'pH paradox' may be an important factor in reperfusion injury to ischemic tissue. 7. In myocytes, amiloride derivatives prevent the pH paradox by blocking  $\text{Na}^{+}/\text{H}^{+}$  exchange producing a rise of intracellular pH. Our working hypothesis is that intracellular acidosis suppresses degradative processes (proteolysis, phospholipid hydrolysis, nucleic acid breakdown) activated by hypoxic and toxic stress. In the pH paradox, inhibition of these pH-dependent degradative processes is removed when acidotic pH is returned to normal, leading rapidly to cell death.

**SIGNIFICANCE:** We have discovered an important new process involved in the pathogenesis of reperfusion injury. Understanding of this pH-dependent process will lead to interventional strategies that can minimize reperfusion injury and permit the rescue of injured myocardium and other tissues.

**WORK PLAN (next 12 months):** Our goal is to reveal the mechanisms underlying the pH paradox in reperfusion injury. Specifically, we will evaluate the interaction of  $H^+$  and  $Ca^{2+}$  ions during the pH paradox. We will test the hypothesis that exchange of intracellular  $H^+$  for extracellular  $Na^+$  secondarily promotes exchange of intracellular  $Na^+$  for extracellular  $Ca^{2+}$  and that the resulting buildup of intracellular  $Ca^{2+}$  leads to mitochondrial  $Ca^{2+}$  overload, hypercontracture and activation of degradative enzymes leading to cell death. We will examine whether calciphor and inhibitors of degradative enzymes (e.g., proteases and phospholipase) can prevent the  $Ca^{2+}$ -initiated events.

**PUBLICATIONS AND ABSTRACTS SUPPORTED ALL OR IN PART BY J-1433 (last 12 months):** 14 journal articles, 7 book chapters, 19 abstracts, 1 letter to the editor, and 6 papers submitted to refereed journals. Representative publications are:

1. Lemasters, J.J., G.J. Gores, A.-L. Nieminen, T.L. Dawson, B.E. Wray and B. Herman (1990) Multiparameter digitized video microscopy of toxic and hypoxic injury in single cells. *Environ. Health Perspect.* **84**, 83-94.
2. Roe, M.W., J.J. Lemasters and B. Herman (1990) Assessment of Fura-2 for measurements of cytosolic free calcium. *Cell Calcium* **11**, 63-73.
3. Nieminen, A.-L., G.J. Gores, T.L. Dawson, B. Herman and J.J. Lemasters (1990) Toxic injury from mercuric chloride in rat hepatocytes. *J. Biol. Chem.* **265**, 2399-2408.
4. Nieminen, A.-L., T.L. Dawson, G.J. Gores, T. Kawanishi, B. Herman and J.J. Lemasters (1990) Protection by acidotic pH and fructose against lethal injury to rat hepatocytes from mitochondrial inhibition, ionophores and oxidant chemicals. *Biochem. Biophys. Res. Commun.* **167**, 600-606.
5. Gores, G.J., B. Herman and J.J. Lemasters (1990) Plasma membrane bleb formation and rupture: a common feature of hepatocellular injury. *Hepatology* **11**, 690-698.
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8. Currin, R.T., G.J. Gores, R.G. Thurman and J.J. Lemasters (1991) Protection by acidotic pH against anoxic cell killing in perfused rat liver: evidence for a 'pH paradox'. *FASEB J.* **5**, 207-210.
9. Harrison, D.C., J.J. Lemasters and B. Herman (1991) A pH-dependent phospholipase  $A_2$  contributes to loss of plasma membrane integrity during chemical hypoxia in rat hepatocytes. *Biochem. Biophys. Res. Commun.* **174**, 654-659.
10. Bond, J.M., B. Herman and J.J. Lemasters (1991) Recovery of cultured rat neonatal myocytes from hypercontracture after chemical hypoxia. *Res. Commun. Chem. Pathol. Pharmacol.* **71**, 195-208.
11. Florine-Casteel, J.J. Lemasters and B. Herman (1991) Lipid order in hepatocyte plasma membrane blebs during chemical hypoxia measured by digitized video fluorescence polarization microscopy. *FASEB J.* **5**, 2078-2084.

## **ANNUAL PROGRESS REPORT FOR 1991**

**GRANT #:** N00014-89-J-1915

**R & T Code** 441q 910

**PRINCIPAL INVESTIGATOR:** Harold Charles Smith

**CONTRACTOR:** University of Rochester

**CONTRACT TITLE:** Smooth Muscle Model System for the Induction of Oxygen Regulation Proteins During Ischemia

**REPORTING PERIOD:** 2 June 1990 - 3 June 1991

**AWARD PERIOD:** 1 July 1989 - 30 June 1992

**RESEARCH OBJECTIVE:** To investigate the response of myogenic cells to low glucose and low oxygen stress in terms of alterations in protein synthesis and the cell cycle.

**ACCOMPLISHMENTS (last 12 months):** (1) Proteins synthesized by myoblasts and myocytes under conditions believed to be operational in ischemia has been examined relative to a shorter exposure to hypoxia (6 h rather than 12) and to low glucose levels. The profile of proteins on autoradiographs of two dimensional gels was found to be qualitatively similar in long and short duration hypoxia but somewhat different in glucose-stressed cells. Specifically, while the synthesis of the major oxygen stress proteins (ORPs) could be seen (though reduced from that typically seen in hypoxic cells) the proliferation specific proteins PSP 100 and PSP 9 were not induced in myocytes. These data suggest that there may be unique cellular responses to the individual parameters in the ischemic microenvironment. (2) Flow cytometric DNA histogram analysis of the cell cycle distribution of myoblasts and myocytes following hypoxia and reoxygenation has revealed that hypoxia-stressed myocytes acquire the ability to over-ride cell contact-inhibition and respond to serum growth factors by re-entering the cell cycle. The effect is demonstrable within 48 h of reoxygenation, requires glucose and is inducible in serum-free media without wounding of the cell syncytium. These data suggest that one aspect of tissue compromise by ischemia can be the modulation of the differentiated cell phenotype. (3) Analysis of the basis for the induction of the proliferative response in myocytes has been initiated by examining the levels of ras activity in these cells in collaboration with Dr. Ian Macara at the University of Rochester. The data show that ras levels are extremely high in myoblasts and that these levels are not altered by differentiation or hypoxia. These data suggest that although ras pathways may be important for signaling; alterations which impart proliferative characteristics on hypoxic myocytes are not primarily due to activation of ras.

**SIGNIFICANCE:** We usually think of tissue and organ failure following ischemia as a primary reflection of cell death. Our work points to the possibility that modulation of the differentiated cell with proliferative characteristics occurs and may have a dramatic affect on tissue function. Our data suggest that different ischemic parameters will "move" the cell through this process by similar as well as different pathways and that the internal signaling will be complex.

**WORK PLAN (the last 12 months):** (1) In the past experiments, cells were made hypoxic in "fresh" media. In this way the effects of "factors" in "spent" media, reduced glucose and reduced pH could be more or less returned to a base line leaving hypoxia as the primary variable. Given the data on the effects of reduced pH from others in the program and our own on the effects of low glucose, it is clear that influence of factors either alone or in concert with hypoxia must be examined. Particularly, we will examine the two dimensional gel profiles of newly synthesized proteins in myoblast and myocytes at various times following the induction of (a) low pH (5.8-6.2) using lactate/HCl to reduce pH, (b) low pH using spent ("conditioned") media, (c) hypoxia with the conditions in "a" and "b" and (d) reoxygenation following the conditions in "c". (2) A collaboration has been established with Dr. John Ludlow at the University of Rochester to measure the amount and phosphorylation of the anti-oncogene protein Rb under the conditions which are of interest in this project. (3) A collaboration with Dr. Frank Lee at the University of Rochester has been established to quantitate the levels of thiols and glutathione-S-transferase under the conditions which are of interest in this project.

**PUBLICATIONS (last 12 months):**

1. D Roll, BJ Murphy, KR Laderoute, RM Sutherland & HC Smith (1991) Oxygen Regulated 80 kDa Protein and Glucose Regulated 78 kDa Protein are Identical. *Molec. Cell Biochem.* 103 in press.
2. AJ Butler, MJ Eagleton, D Wang, RL Howell, AR Strauch, V Khasgiwala & HC Smith (1991) Induction of the Proliferative Phenotype in Differentiated Myogenic Cells by Hypoxia. *J. Biol. Chem.* (accepted)
3. JW Backus, MJ Eagleton, SG Harris, CE Sparks, JD Sparks & HC Smith (1991) Quantitation of Endogenous Liver Apolipoprotein B mRNA Editing. *Biochem. Biophys. Res. Commun.* 170 513-518.
4. HC Smith, S-R Kuo, JW Backus, SG Harris, CE Sparks & JD Sparks (1991) In vitro Apolipoprotein B mRNA Editing: Identification of a 27S Editing Complex. *Proc. Natl. Acad. Sci. (USA)* 88 1489-1493.



## ANNUAL PROGRESS REPORT

GRANT #: N00014-88-K-0427

R&T CODE: 441q802

PRINCIPAL INVESTIGATOR: Benjamin F. Trump, M.D.

INSTITUTION: Univ. of Maryland at Baltimore, Dept. of Pathology

GRANT TITLE: Altered Signal Transduction in Renal Cell Injury  
Following Hemorrhagic Shock or Anoxia

REPORTING PERIOD: 01 June 1990 - 31 May 1991

AWARD PERIOD: 01 July 1987 - 30 June 1991

OBJECTIVE: To investigate anoxia, ischemia, & inflammatory cell injury on various *in vivo* & *in vitro* animal models with emphasis on ion deregulation & its effects on transmembrane signalling & gene expression.

ACCOMPLISHMENTS: 1. Modification of  $[Ca^{2+}]_i$ ,  $pH_i$ , &  $[Mg^{2+}]_i$ : Using a PTE model *in vitro*, we determined that very early increases occur in  $[Ca^{2+}]_i$ , from influx from the EC space (Ca ionophores and complement, oxidative injury from X/XOD), redistribution from IC stores (SH agents), & both influx & redistribution including  $HgCl_2$ , & models of anoxia. Also observed were increases of  $pH_i$  (0.1 unit) after KCN or KCN + IAA, while  $HgCl_2$  produced a decrease (0.6 units), the recovery of which was amiloride-insensitive, indicating no involvement of  $Na^+/H^+$  exchange. Similar changes, but less were seen with Ca ionophores & followed by amiloride-sensitive re-alkalinization. Marked increases (2.5 fold) of  $[Mg^{2+}]_i$  were observed with KCN or KCN + IAA, slight decreases with  $HgCl_2$ , & steady increases with Ca ionophores. 2. Role of Ca-activated proteases: Activation of calpains by modification of cellular SH groups using  $HgCl_2$  caused early blebbing & marked changes in cytoskeletal elements, (F-actin). Modification of Ca proteases using antipain & leupeptin, showed amelioration of bleb formation, actin disruption, & cell killing. 3. Complement-induced injury: A significant role for  $Ca^{2+}$  influx in mediating subsequent changes in ATP-synthesis & subsequent cell injury & death was found, namely the dramatic & extremely rapid increase in  $[Ca^{2+}]_i$  (less than 30 sec), followed by decreased mitochondrial membrane potential, loss of ATP synthesis, & irreversible change. 4. Oxidative stress-induced injury: Ca-dependent cell killing began rapidly after the addition of X/XOD (500  $\mu M$ /25 mU/ml) with  $[Ca^{2+}]_i$  rising within sec. This increase was eliminated in low  $Ca^{2+}$  medium (EGTA). Initial changes could be reversed using pulse exposures of X/XOD followed by oxidant-free media. 5. Oxidant stress-induced immediate, early, and stress genes: After X/XOD,  $[Ca^{2+}]_i$  increased, followed by a rapid & transient c-fos gene expression. This response was ameliorated by reduction of  $Ca^{2+}$  influx using  $Ca^{2+}$ -free EGTA- containing media. X/XOD-induced DNA SS breaks were also ameliorated by preventing  $Ca^{2+}$  influx. We found no evidence that DNA SS breaks stimulated fos expression by stimulating poly-ADP ribosylation, but did find that protein kinases including PKC are involved. 6. Advances in technology: We further developed ancillary imaging equipment, including modification of a chamber for controlled anoxia-reperfusion studies. An AT-based imaging work station was installed as were photon counters.

SIGNIFICANCE: By characterizing these effects, we will be able to extrapolate animal data to man & thus achieve a better understanding of the mechanisms involved in shock, trauma, & related injuries.

WORK PLAN: 1. Complete  $[Ca^{2+}]_i$  studies, using DIFM, including the reversibility of alterations in  $[Ca^{2+}]_i$  during anoxia (argon gas or KCN) without substrate (glucose) followed by reoxygenation & restoration of substrate. 2. Complete in vitro studies on gene expression by characterizing immediate early genes following anoxia. 3. Continue studies on the role of stress proteins in vitro in anoxia without substrate & oxidative stress by exploring the mechanisms as they relate to  $[Ca^{2+}]_i$  & immediate early genes. 4. Extend studies on pH to DIFM studies of monolayers exposed to conditions of anoxia & anoxia without substrate, as well as to oxidative stress. 5. Begin protection studies by investigating: a) the mechanisms of  $[Ca^{2+}]_i$  increases & b) the protection afforded by limiting increases in  $[Ca^{2+}]_i$  & by EC acidosis. 6. Begin in vitro studies on  $[Mg^{2+}]_i$  using DIFM & monolayers. 7. Begin investigating the role of actin by observing morphological changes, particularly blebbing, following ischemia & reflow injury. Micro-inject monolayer cells with fluorescent Ab to actin or with fluorescent phalloidin derivatives to visualize alterations in actin microfilaments. 8. Begin studies of gene expression in shock & ischemia in an in vivo clamp/reflow model using probes for c-fos, c-myc, hsp72/73 & gadd153 to investigate the kinetics of altered expression of immediate early & stress genes following both ischemia & reflow injury.

PUBLICATIONS AND ABSTRACTS:

A. Published:

Papers (reprints enclosed)

Cerutti, P.A. and Trump, B.F. (1991) Inflammation and oxidant stress in carcinogenesis. *Cancer Cells* 3: 1-7. Trump, B.F. and Berezesky, I.K. (1990) The importance of calcium regulation in toxic cell injury. *Studies utilizing the technology of DIFM. Clinics in Laboratory Medicine* 10: 531-547. Trump, B.F. et al. (1990) Nephrotoxicity in vitro: Role of ion deregulation in signal transduction following injury--Studies utilizing digital imaging fluorescence microscopy. *Toxic. In Vitro* 4: 409-414. Trump, B.F. et al. (1990) Studies of human bronchial epithelium in vitro: Changes of  $[Ca^{2+}]_i$  in relation to injury, growth and differentiation. *Toxic. In Vitro* 4(4/5): 646-653. Trump, B.F. et al. (1990) Cell toxicity and ion regulation in the bronchus and kidney: An hypothesis. *Basic Science in Toxicology: Proc Vth Internatl Congr of Toxicol*, pp. 636-650. Trump, B.F. et al. (1990) Relation between toxicity and carcinogenesis in the kidney: An heuristic hypothesis. *Renal Failure* 12: 183-191. Smith, M. et al. (1991) Cytosolic  $Ca^{2+}$  deregulation and blebbing after  $HgCl_2$  injury to cultured rabbit proximal tubule cells as determined by digital imaging microscopy. *Proc Natl Acad Sci USA* 88:4926-4930. Swann, J.D. et al. (1991) Oxidative injury induces influx-dependent changes in intracellular calcium homeostasis. *Toxicol. Pathol.* 19(2): 128-137. Papadimitriou, J.C. et al. (1991) Quantitative analysis of adenine nucleotides during prelytic phase of cell death mediated by C5b-9. *J. Immunol.* 147:212-217.

\*Because of space constraints, 11 published abstracts and 1 chapter plus 4 submitted papers are not listed.

## ANNUAL PROGRESS REPORT

GRANT #: NO0014-90-J--1893

R&T CODE: 4414033

PRINCIPAL INVESTIGATOR: Roy A. Tassava

INSTITUTION: The Ohio State University

GRANT TITLE: Breakdown of the ST1 antigen correlates with the events of epimorphic regeneration in salamanders: Extension of a model system to wound healing in frogs and mice.

REPORTING PERIOD: 1 July, 1990 - 31 May, 1991 (11 months).

AWARD PERIOD: 1 July, 1990 - 30 June, 1993.

OBJECTIVE: To purify the ST1 antigen to homogeneity and biochemically characterize and identify this protein; to make polyclonal Ab and molecular probes to examine ST1 in wounds and amputated limbs of frogs and mice; to begin to understand the mechanisms and relevance of ST1 breakdown during limb regeneration and wound healing.

ACCOMPLISHMENTS: We have established a method that consistently extracts the ST1 antigen. In Western Blots of salamander limb soft tissues extracted with 6 M guanidine-HCl, 3 major bands with Mr ( $\times 10^3$ ) of 130, 210, and 230 are revealed. The ST1 antigen in these extracts is not sensitive to heparinase 11 or 111, hyaluronidase, or chondroitinase ABC, is partially sensitive to sodium m-periodate, and is completely sensitive to trypsin and collagenase. The ST1 antigen is thus likely a collagen and we are pursuing that possibility (Yang et al., submitted; Yang et al., in preparation).

We have shown that the breakdown of the ST1 antigen that occurs early in regeneration at the limb tip is nerve independent but partially wound epithelium dependent (Yang et al., submitted).

We have shown that the wound epithelium in salamanders is a complex, multifunctional tissue that is essential for regeneration. The wound epithelium plays a role in breakdown of the ST1 antigen early in regeneration and therefore we have worked hard at characterizing this tissue in terms of its function and biochemistry. We know by use of mAbs and in situ hybridization that the wound epithelium expresses the WE3, WE4, and WE5 antigens (Castilla and Tassava, submitted). and expresses the gene for tenascin (Onda et al., submitted). Most recently, we have shown that synthesis of the WE3 antigen in the wound epithelium is enhanced by retinoic acid (Tassava, in preparation).

We have carried out an extensive characterization of reactivity of a number of mAbs (ST1, WE3, WE4, MT1, MT2) in superficial and deep flank wounds. These data are being prepared for publication (Castilla and Tassava, in preparation).

SIGNIFICANCE: Establishing a protocol that consistently extracts the ST1 antigen is an important result and will lead to identification of this matrix protein (see below). Studies of ST1 with non-regenerating species, i.e. frogs and mice, will now be possible. Our data showing that the wound epithelium is partially responsible for ST1 breakdown adds another dimension to the many biochemical and functional roles that this critical tissue type displays in regenerating species. A number of proteins are synthesized by the wound epithelium but not by epidermis, including the WE3, WE4, and WE5 antigens as well as tenascin, attesting to the multifaceted nature of the wound epithelium. Whether this array of proteins is synthesized by wound epithelium of flank wounds and amputated limbs in non-regenerating species will be important to determine.

WORK PLAN (next 12 months): Now that we can extract the ST1 antigen and, given the biochemical data available, we can obtain more monoclonal Abs as well as polyclonal Abs. With these additional Abs, we will: (1) probe a cDNA expression library to obtain molecular "sequence" information and a molecular probe for in situ hybridization and, (2) use the Abs to examine whether or not the ST1 antigen breaks down in flank wounds and amputated, non-regenerating limbs of frogs and mice, a major goal of the proposal. With a tenascin riboprobe specific for frogs (gift), we will examine wound epithelium and mesenchyme of frog flanks and limbs for tenascin gene expression. We anticipate that the pattern and extent of expression by both cell types will differ markedly from salamanders, i.e. regenerating species. Now that we know that the WE3 antigen is markedly up-regulated in the wound epithelium by retinoic acid, we are using extracts of these WE3 rich wound epithelia to immunize mice to obtain additional mAbs against the WE3 antigen and other potentially interesting wound epithelial antigens.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Castilla, M. and R.A. Tassava (1991) Extraction of the WE3 antigen and comparison of reactivities of mAbs WE3 and WE4 in regenerate epithelium and body tissues. Submitted.
2. Onda, H., M.L. Poulin, R.A. Tassava, and I.M. Chiu (1991) Characterization of a newt tenascin cDNA and localization of tenascin mRNA during newt limb regeneration by in situ hybridization. Submitted to Devel. Biol.
3. Yang, E.V., D.T. Shima, and R.A. Tassava (1991) Monoclonal antibody ST1 identifies an antigen that is abundant in the axolotl and newt limb stump but is absent from the undifferentiated regenerate. Submitted.

**PRESSURE BIOLOGY**

**CORE PROGRAM**

SCIENTIFIC OFFICERS: Drs. Harold Bright, Jeannine Majde,  
Constance Oliver and Igor Vodyanoy

BEGAN: MARCH 1, 1987

PROGRAM OBJECTIVE: TO INVESTIGATE THE MACROMOLECULAR, MEMBRANE, AND SIGNAL TRANSDUCTION RESPONSES TO INCREASED HYDROSTATIC PRESSURES.

NAVY OBJECTIVE: TO UNDERSTAND FUNDAMENTAL CELLULAR EVENTS IN DIVING PHYSIOLOGY.

## ANNUAL PROGRESS REPORT

GRANT#: N00014-88-K-0324

R&T CODE:4415807

PRINCIPAL INVESTIGATOR: P. A. George Fortes

INSTITUTION: University of California, San Diego

GRANT TITLE: Effects of Pressure on the Conformational Dynamics of (Na,K)-ATPase

REPORTING PERIOD: 1 July 1989 - 31 May 1991

OBJECTIVE: To understand the effects of hydrostatic pressure on (Na,K)-ATPase at the molecular level.

ACCOMPLISHMENTS: We developed a new coupled enzyme assay to measure ATPase activity under pressure in the absence of potassium. This assay uses phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase as the coupling enzymes. The older methods use pyruvate kinase, which has an absolute requirement for potassium. The new method has allowed us to study the effect of pressure on sodium-dependent ATPase activity. The pathway of the Na-ATPase reaction appears to be the same as that of the (Na+K)-ATPase reaction, except for the K-dependent steps. This Na-ATPase activity is associated with electrogenic sodium transport in intact cells.

We discovered that pressure (100-500 bar) stimulates the Na-ATPase activity up to 500%. By contrast, pressure strongly inhibits the (Na+K)-ATPase activity. We have been investigating the mechanisms of this paradoxical behavior. So far the kinetic data on the dependence of the pressure responses on ion concentrations and their sensitivity to oligomycin are consistent with the notion that enzyme intermediates that have bound or occluded ions are stabilized by pressure because they have a decreased volume. This hypothesis accounts for both the stimulation and the inhibition of activity by pressure that we have observed, because deocclusion of potassium is rate-limiting, whereas sodium binding accelerates enzyme turnover. The pressure dependence of the potassium binding equilibria measured by changes in fluorescence of site-directed probes also suggest that the enzyme forms that have occluded potassium are favored by pressure.

We have used the voltage-sensitive dye RH-421 to study charge movements within the enzyme as a function of pressure. We initiate the reaction by flash photolysis of caged ATP. So far, the data suggest that pressure inhibits Na dissociation from the enzyme. This method may allow us to measure the pressure dependence of the rate constants of

conformational transitions.

Dr. Richard Aguilar completed his independent study project required for the M.D. degree studying interactions of ligands and pressure on the distance between a fluorescein labelled cys residue and TNP-ATP bound to the active site. No detectable distance changes were seen with pressure up to 2 kbar.

**SIGNIFICANCE:** The understanding of the effects of pressure on the conformational transitions of (Na,K)-ATPase will contribute to the elucidation of the molecular mechanism of ion pumps and may have general implications on cell physiology in, for example, deep sea environments. Some of the enzyme intermediates that are stabilized by pressure are the ones that bind cardiac glycosides, like ouabain. Since ouabain seems to be an endogenous hormone, our findings have implications on possible inotropic effects of pressure on divers.

**WORK PLAN:** Next year we intend to measure the effect of pressure on cardiac glycoside binding, and on the rate constants of phosphorylation and phosphoenzyme transitions, as outlined in my original proposal. The use of photoactivatable substrates will allow us to circumvent optical artefacts due to pressure changes, since we can initiate the reaction after equilibration at the desired pressure. We will attempt also to measure changes in subunit interactions and, if we can develop suitable probes, to study the nucleotide sites.

#### PUBLICATIONS AND REPORTS

We have several manuscripts in preparation. The following abstracts have appeared:

1. Fortes, P.A.G. (1990). The Use of Hydrostatic Pressure to Study Conformational Transitions in (Na,K)-ATPase. Abstracts. 2nd. Congr. Int. de Biofisica del Cono Sur. Punta de Tralca, Chile, p. 27.
2. Fortes, P.A.G. (1991). Paradoxical Effects of Hydrostatic Pressure on (Na,K)ATPase: Evidence that Intermediates with Occluded Cations Have Decreased Volume. Biophys. J. 59:561a
3. Schwappach, B., Gassman, W., & Fortes, P.A.G. (1991). Interaction of the Voltage-Sensitive Dye RH-421 with (Na,K)-ATPase. Biophys. J. 59:339a.

## PRESSURE STUDIES OF PROTEIN DYNAMICS

ONR Contract: N0014-89-J-1300

R&T Code:4413015

Principal Investigators: Hans Frauenfelder and Robert D. Young

Institution: University of Illinois at Urbana-Champaign

Reporting Period: 1 March 1990 to 28 February, 1991

Award Period 1 March 1989 to 28 February, 1992

1. Goals: A quantitative understanding of the relations among protein structure, motions, and function.

2. Approach: Our functional tool is ligand binding to heme proteins. Our experimental tools involve flash photolysis over wide ranges in time (10 ns to 10 ks), temperature (5 to 330K), viscosity (1 to 10<sup>5</sup> centipoise), and pressure (1 to 3000 atm.). Pressure is essential as static variable and as dynamic perturbation to initiate protein relaxations. The infrared stretch bands of carbon monoxide have proved to be valuable local markers of protein reactions and relaxations.

3. Brief Summary of Results: Progress has been very good. We now have experimental evidence that protein relaxation and fluctuations are crucial to control and specificity in ligand binding to heme proteins. We have also shown that proteins possess a hierarchical arrangement of conformational substates and motions. The hierarchy extends through at least three tiers. The research funded under this contract has been essential in establishing this dynamic model of protein function. We briefly summarize the main results:

3.1 Hierarchy of Substates and Motions in Proteins: We use pressure-jump near the transition temperature to probe relaxation processes in various heme proteins, including myoglobin (Mb), horseradish peroxidase (HRP), cytochrome P450 (P450), and chloroperoxidase (CPO) with CO as ligand. We monitor the infrared CO stretch bands as function of time and temperature. The pressure-release studies show that each of the proteins has at least two tiers of conformational substates (CS0 and CS1) and motions (FIM0 and FIM1). The details of the hierarchy depend on the protein structure. Both tiers have a strong dependence on the viscosity of the solvent indicating that they involve large parts of the protein.

3.1 Time and Temperature Dependence of Relaxation in Proteins: All of the proteins studied show relaxation process which is nonexponential in time and non-Arrhenius in temperature as also seen for other complex systems such as glasses and spin glasses. The time dependence of FIM1 can be described by a stretched exponential, FIM0 is much closer to a exponential.

3.3 Protein Dynamics and Reactions - Ligand Binding to Heme Proteins: Our studies of the binding of CO and O<sub>2</sub> to myoglobin and other monomeric heme proteins have resulted in a model of the binding process in which protein relaxation and fluctuations are essential for control and specificity. Our model unifies a large body of experimental results and makes predictions that can be tested. The pressure studies of protein relaxations were essential to development of the model; they show that Mb has a third tier of conformational substates (tier 2) and motions (FIM2) which are local and probably involve the structure of the proximal histidine, iron, and porphyrin. The results show that ligand binding to heme proteins is controlled at the proximal side by the



barrier for bond formation at the heme iron and that protein relaxations and fluctuations control the barrier as well as entry and exit from the globin matrix. Discrimination between CO and O<sub>2</sub> is determined by the distal side.

4. Work Plan We will continue our evaluation and analysis of the voluminous data set for each of the heme proteins. The rich spectrum of motions observed promises to yield important information regarding the conformational substates and motions in heme proteins. We intend to explore further two aspects of the results: (i) The *elastic shift* which occurs faster than our shortest time scale after pressure release. (ii) The *relaxation FIMX* which is exponential in time. It occurs in each of the heme proteins and may be related to tier 2 of substates. We also will continue to develop a system for fast (millisecond) pressure release so as to extend our time range by three orders of magnitude to faster times.

#### 5. Publications

1. P.J. Steinbach, A. Ansari, J. Berendzen, K. Chu, B.R. Cowen, D. Ehrenstein, H. Frauenfelder, J.B. Johnson, D.C. Lamb, S. Luck, J.R. Mourant, G.U. Nienhaus, P. Ormos, R. Philipp, A.-H. Xie, R.D. Young, "Ligand binding to heme proteins: The connection between function and dynamics," Biochemistry, Vol. 30, 3488 (1991).

2. M.K. Hong, D. Braunstein, B.R. Cowen, H. Frauenfelder, A.E.T. Iben, J.R. Mourant, P. Ormos, R. Scholl, A. Schulte, P.J. Steinbach, A.-H. Xie, & R.D. Young, "Conformational substates and motions in myoglobin: External influences on structure and dynamics," Biophysical Journal Vol. 58, 429 (1990).

3. R.D. Young & R. Scholl, "Proteins as complex systems," Journal of Non-Crystalline Solids in press (1991)

4. R. Young, "Glassy dynamics and relaxation in proteins." In Relaxation in Complex Systems and Related Topics, Ed. I.A. Campbell and C. Giovannella, Nato ASI Series B: Physics Vol. 222, 185 (1990).

5. R. Scholl, S.R. Blanke, K. Chu, D. Ehrenstein, H. Frauenfelder, L.P. Hager, J.B. Johnson, C. Jung, J.R. Mourant, R. Philipp, S.G. Sligar, P. Stayton, Y.J. Suh, & R.D. Young, "Relaxation in carbonmonoxy heme proteins." Biophysical Society, Baltimore MD, February 1990 Abstract: Biophysical Journal, Vol. 57, 233a (1990)).

6. H. Frauenfelder, G. U. Nienhaus & J. B. Johnson, "Rate Processes in Proteins." Ber. Bunsenges. Phys. Chem. Vol. 95, 272 (1991).

7. H. Frauenfelder, K. Chu & R. Philipp, "Physics from Proteins." In press (1991).

## ANNUAL PROGRESS REPORT

GRANT#: N00014-89-J-3009

R&T CODE: 4415912

PRINCIPAL INVESTIGATOR: Herbert R. Halvorson

INSTITUTION: Henry Ford Hospital

GRANT TITLE: Effects of Pressure on Calcium Binding to  
Brain Protein

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 Sep 1989 - 31 Aug 1992

OBJECTIVE: To investigate the pressure-sensitivity of interactions between the regulatory protein calmodulin and target proteins or analogues of the target proteins. The purpose is to determine if the pressure-dependency of calcium binding to calmodulin might possibly underlie the high-pressure nervous syndrome (HPNS).

ACCOMPLISHMENTS (last 12 months): We have demonstrated a significant sensitivity to pressure of the interactions between calmodulin and the peptide melittin, a sensitivity that depends on calcium concentration. The effects occur at physiological  $\text{Ca}^{2+}$  concentration and at pressures associated with HPNS (50 bar), although the model and its concentrations are certainly not physiological. The magnitudes of the effects are consistent with a significant physiological response. A major difficulty has been coping with an uncertain stoichiometry for the binding of melittin to calmodulin: additional copies of melittin bind at melittin/calmodulin ratios greater than one. The problem is avoided simply by working at low melittin concentrations.

SIGNIFICANCE: The preliminary analysis of calmodulin-melittin interactions reveals a pressure effect (140 mL/mole volume change) large enough to be physiologically significant at 50 bar. This is in accord with the original hypothesis that linkage between metal ion binding and protein-protein interaction could be a cause of some physiological effects of hydrostatic pressure.

WORK PLAN (next 12 months): In the coming year we plan to move to a more physiological "target", probably myosin light-chain kinase. The primary concern is obtaining sufficient material of the target. Because the experiment is intrinsically insensitive, several mg of protein are required. The appropriate controls (effects of calcium, pressure and ANS in the absence of calmodulin) will be performed.

We are preparing the work already done (the melittin analogue study) for publication. This may entail some additional experiments on the system.

PUBLICATIONS AND REPORTS (last 12 months): Two chapters were prepared for a forthcoming volume of "Methods in Enzymology" devoted to numerical methods (copies enclosed). Likely publication date is late 1991.

1. H R Halvorson, The use of programs for symbolic mathematics in biochemistry.
2. H R Halvorson, The Pade-Laplace algorithm for sums of exponentials: Selecting an appropriate exponential model and initial estimates for exponential fitting.

## Annual Progress Report

**Grant #:** N00014-88-K-0550

**R&T Code:** 4414802

**Principal Investigator:** Perry M. Hogan, Ph.D.

**Institution:** Department of Physiology  
State University of N.Y. at Buffalo

**Grant Title:** Cellular and Molecular Mechanisms of High Pressure  
Inotropy in Cardiac Muscle

**Reporting Period:** 1 August 1990 - 3 June 1991

**Award Period:** 1 August 1990 - 31 July 1991

**Objective:** The objective of this project is to fully exploit the isolated cardiac myocyte as a model system for investigating the effects of high hydrostatic pressure on calcium regulated cellular phenomena.

**Accomplishments** (last 10 months): To measure myocyte performance simultaneously with intracellular calcium we have development a high speed video, line scan camera using a linear photodiode array (PDA). In this reporting period we have completed work on the PDA camera and have begun testing its performance and that of the FURA-2 calcium assay system described in a previous report.

PDA camera for sarcomere measurements: This device will be used to measure myocyte contraction while the cell is exposed to elevated hydrostatic pressure. A differential interference contrast (DIC) image of the myocyte is projected upon a 1024 element linear photodiode array attached to a side port of the microscope. The pixels are spaced on 25 micron centers and each is 2.5 mm wide. The image is aligned so that the long axis of each pixel is parallel with the banding pattern of the sarcomeres. In this way, each sarcomere covers several pixels, the exact number depending upon the magnification used and the instantaneous length of the sarcomere itself. At 100x magnification the spatial frequency of the photosensors referenced to the objective plane is 4.34 pixels/micron. A sequence of images are scanned, digitized, and stored in a computer-based frame buffer. The unloaded, isotonic contraction is reconstructed from this set of images. In its present configuration the PDA camera can capture myocyte images at 1 millisecond per image and operates in tandem with the with FURA-2 assay system without degrading the performance of either system. A flash converter and frame buffer are used to control the photodiode array electronics and to transfer the frame data into computer memory. The frame buffer can store up to 2048 sequential frames transferred at speeds up to 8,000,000 bytes/sec.

Calibration and testing of the FURA-2 calcium assay system: In our FURA 2 calcium measuring system, wavelength switching is accomplished using a variable speed spinning sector wheel fitted with a pair of aperture masking, adjustable slits. During operation, the combination of wheel speed and slit width determines

aperture and switching times and thus sampling rate. Slit width is adjusted to mask a region of interest containing the cell under study. Slit width (4.3 to 30.2 microns) and wheel speed (20 to 100 rps) are continuously adjustable to meet the requirements of the specific experiment. Aperture time varies between 747 and 122 microseconds - values that correspond to calcium sampling rates of 1.2 to 6.0 samples/millisecond. Further, the dynamic range of FURA-2 is not degraded at these fast sampling rates.

System software revisions: UVSYS, the software operating system developed for this project, has been revised for seamless integration of the PDA camera. Further, the software has been extended to include a number of system utilities for managing data files. Finally, we have added a light meter function that facilitates trouble-shooting, tuning and calibrating the instrument. The light meter function utilizes the same system resources used for collecting data.

**Significance:** Cellular functions regulated by calcium appear highly sensitive to changes in hydrostatic pressure. It is now possible, using the tools developed in this project, to critically assess the mechanisms underlying this relationship. Clearly, the identification of a link between high pressure and the second messenger properties of calcium would have important and wide spread implications for cellular regulation. In a practical sense, clarification of this critical issue will certainly contribute to the development of a rational strategy for ameliorating the deleterious effects of elevated hydrostatic pressure.

**Work plan** (next 12 months): After calibrating FURA-2 for use at high hydrostatic pressure, experiments will be conducted to determine the basic relations between resting cytosolic calcium, calcium transients associated with twitch contractions, and elevated hydrostatic pressure in mammalian cardiac myocytes. In all cases, sarcomere spacing will be correlated with changes in cytosolic calcium. Based on previous findings we suspect that the positive inotropic effect of high hydrostatic pressure involves alterations in activity of the (Na,K) pump-(Na,Ca)-exchange complex operating across the sarcolemma and/or the Ca-pump operating across the membranes of the sarcoplasmic reticulum. Accordingly, experiments will be conducted to test for the effects of pressure on these dynamic elements in the intact cell.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-88-J-1108

R&T CODE: 4414803

PRINCIPAL INVESTIGATOR: Richard B. Philp, D.V.M., Ph.D.

INSTITUTION: Dept. of Pharmacology & Toxicology,  
The University of Western Ontario,  
London, Canada

GRANT TITLE: Effects of Pressure and Anesthetics, Including Inert  
Narcotic Gases, on Cell Membranes and Release Mechanisms

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 June 1988 - 31 May 1991

OBJECTIVE: To improve our understanding of the effects of inert narcotic gases at raised partial pressures, and of pressure per se on cell function, particularly at the membrane level, and to determine why hydrostatic pressure and narcosis are mutually antagonistic.

ACCOMPLISHMENTS (last 12 months): Our previous investigations showed that elevated pressures of He and N<sub>2</sub> (18-36 ATA) inhibited platelet aggregation induced by agents requiring external Ca<sup>2+</sup> but not by those lacking this requirement. Since it was not possible to distinguish pressure effects from narcotic effects (both being present when N<sub>2</sub> was the compression gas), we have now completed studies of elevated pressure (to 6.4ATA) of xenon (Xe) and nitrous oxide (N<sub>2</sub>O). Xe is 40x more potent narcotically than N<sub>2</sub> and it is chemically inert. 1 ATA of Xe thus equates narcotically with 40 ATA of N<sub>2</sub>. N<sub>2</sub>O is equinarcotic with Xe but it is classified as an anesthetic/analgesic. While N<sub>2</sub>O inhibited calcium-dependent platelet aggregation in a dose-dependent manner, Xe actually potentiated aggregation. When phorbol myristate, which acts independently of external Ca<sup>2+</sup>, was the aggregation stimulus, both gases were inhibitory. Thus despite their similar narcotic activity, these two gases have different pharmacological characteristics. Xe had opposite effects to those of pressure (on calcium-dependent aggregation) and this fits the pattern of mutual antagonism. It would appear that in this cell system at least, inert gas narcosis is not identical to anesthesia.

Aggregation also was studied in single cell suspensions of the marine sponge Microciona prolifera. When placed in calcium/magnesium-free seawater, cells of this species leave their matrix and become a single cell suspension. When Ca<sup>2+</sup> is added back, the cells aggregate much like human blood platelets. This involves the presence of a species-specific glycoprotein aggregating factor (secreted by the cells) and requires extracellular Ca<sup>2+</sup>. This aggregation was inhibited by 18-36 ATA of He and N<sub>2</sub>, by N<sub>2</sub>O, and was not inhibited by Xe. There was a trend toward potentiation by Xe but it was not statistically-significant. These data form the basis of a paper to be submitted to BBA.

In October of 1989 we took delivery of a small, custom-designed pressure chamber which fits a Hitachi Model 4010 fluorescence spectrometer. With this instrument we hoped to be able to study changes in cytosolic free calcium ([Ca<sup>2+</sup>]<sub>i</sub>) in ADP-stimulated human blood platelets under various conditions of pressure and gases. We undertook the design and testing of the reagent injection system ourselves, and this has consumed considerably more time than was anticipated. However, a system is now in place and data is being accumulated. Using fura-2 loaded platelets we have shown that N<sub>2</sub>O at 6.4, but not 3.7, ATA

significantly ( $p < 0.001$ ) inhibited the ADP-induced increase in  $[Ca^{2+}]_i$  which was  $335 \pm 58.85$  nM as compared to a mean control value of  $806 \pm 110.99$  nM. Various technical problems with this system have now been overcome and it is being used successfully at pressures up to 36 ATA of He to yield reliable data.

**SIGNIFICANCE:** Demonstration that two cell preparations from opposite ends of the phylogenetic spectrum respond in a remarkably similar manner to elevated pressures of gaseous anesthetics and inert gases, and to hydrostatic pressure, strongly suggests that excitable cells possess a common target for these effects. The prominent role of calcium, especially its interaction with adhesion proteins and glycoproteins, makes it an attractive candidate for the target site.

Xe and  $N_2O$  are both narcotic gases but the former is truly "inert". That they have opposing effects on calcium-dependent cell aggregation does not argue in favor of inert gas narcosis being simply a manifestation of anesthesia.

If the rather pronounced inhibition of platelet aggregation by pressure should occur in vivo, there could be impairment of hemostasis in divers exposed to these pressures.

**WORK PLAN:** In the forthcoming year our efforts will be directed toward exploring the hypothesis that the effects of pressure vs. inert gases can be explained by their different actions on calcium-mediated phenomena, in part through influencing calcium binding to reactive sites on proteins and glycoproteins. To this end we will be studying the effects of elevated pressures of He,  $N_2$ , Xe and  $N_2O$  on  $[Ca^{2+}]_i$  of ADP-stimulated, fura-2 loaded, human blood platelets. We should then be able to correlate this with observed effects on aggregation. Similar studies may be attempted with sponge cells, depending on whether a suitable calcium chelator can be found; we have not been able to load them with fura-2. In the future, we hope to study these parameters in another mammalian cell type, possibly cultured astrocytoma cells which may be more relevant to the CNS.

We will also be looking for means to study calcium-protein interactions under pressure in an effort to separate this site from other potential targets such as calcium channels. Several approaches will be tried in order to identify the most productive approach. One technique that is available to us is pressure-tuned vibrational infrared spectroscopy. This technique was developed by P.T.T. Wong at the Steacie Institute of Molecular Science in Ottawa. Dr. Wong has an adjunct professorship in this Department and is a willing collaborator in departmental research.

**PUBLICATIONS AND ABSTRACTS (last 12 months):**

Philp, R.B. Effects of pressure and narcotic gases on aggregation of human blood platelets and marine sponge cells. Proc. II<sup>nd</sup> Internat. Meeting on High Pressure Biol. Toulon, France, Aug. 1990.

McIver, DJL, Philp, RB and Schurch, S. The effects of high pressure on the surface thermodynamics and structure of axisymmetric fluid interfaces (Abst.) *ibid*.

Responses of excitable cells to pressure, inert narcotic gases ( $N_2$ , Xe) and a gaseous anesthetic ( $N_2O$ ) do not suggest a common mechanism. Undersea Biomed. Res. (Abst.) in press. Presented at a workshop on inert gas narcosis, Defence and Civil Inst. of Environmental Med., Downsview, Ont., Oct. 1990.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1865

R&T CODE 4414810

PRINCIPAL INVESTIGATOR: Joseph F. Siebenaller

INSTITUTION: Louisiana State University

GRANT TITLE: Effects of Pressure on Membrane-Associated Receptors and Effector Elements

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 July 1988 - 30 June 1991

OBJECTIVE: To investigate the effects of moderate hydrostatic pressures on signal transduction we have used the A<sub>1</sub> adenosine receptor - inhibitory G protein (G<sub>i</sub>) - adenylyl cyclase system in two species of scorpaenid fish which have served as a model for the study of pressure adaptation. These species, Sebastolobus alascanus and S. altivelis, have similar body temperatures, but dwell at different depths, and thus experience different hydrostatic pressures. The experiments are designed to identify and define at the molecular level the effects of pressure on the components of the signal transduction system in isolation and on the entire functional complex.

ACCOMPLISHMENTS (last 12 months): We have completed work characterizing the effects of pressure on the coupling of the A<sub>1</sub> adenosine receptor in brain membrane preparations (Siebenaller and Murray, 1990; Siebenaller et al., 1991). For the teleost brain membrane preparations, incubation at 5°C and 476 atm does not result in loss of adenylyl cyclase activity or coupling to the A<sub>1</sub> adenosine receptor on subsequent assays at atmospheric pressure. In contrast, rat brain membrane preparations lost 59% of their activity under these conditions.

At atmospheric pressure, the K<sub>m</sub> of 2-deoxy-ATP was identical for the Sebastolobus species adenylyl cyclase. Increased pressure increased the K<sub>m</sub> values in both species. However, the K<sub>m</sub> of 2-deoxy-ATP was less sensitive to pressure for the enzyme from the deeper-living S. altivelis. Basal adenylyl cyclase activity and the inhibitory effect of 100 μmol l<sup>-1</sup> CPA were assayed at 1, 136 and 408 atm. Increased pressure inhibited basal adenylyl cyclase activity in both species. Basal adenylyl cyclase in brain membranes from the rat and 5 additional teleost species were also inhibited by increased pressure. At 136 atm CPA inhibited the adenylyl cyclase from both Sebastolobus species. However, at 408 atm the efficacy of CPA was reversed for S. altivelis, resulting in a stimulation of adenylyl cyclase



activity. The phospholipid and fatty acid contents of brain membranes from the two Sebastolobus species do not differ. [<sup>32</sup>P]ADP-ribosylation by pertussis toxin results in a 10- to 15-fold greater labeling of 39 and 41 kDa G-protein  $\alpha$  subunits in brain membranes of S. altivelis. The G protein complement of these species may play a role in the differential pressure-sensitivity of signal transduction. To assess this possibility, studies are currently underway further characterizing the susceptibility of G proteins in the two species to [<sup>32</sup>P]ADP ribosylation, the content of immune reactive  $\alpha$  and  $\beta$  subunits are being determined, and the effects of hydrostatic pressure on the intrinsic GTPase activity of G protein  $\alpha$  subunits.

SIGNIFICANCE: The effects of hydrostatic pressure on membranes and membrane-associated systems may be the most extensive adaptational problems posed for organisms invading the deep sea. The general importance of pressure perturbation on membranes is seen in the high pressure neurological syndrome which has been demonstrated in a variety of taxa, including fishes. Our experiments indicate that pressure likely affects the enzyme effector and/or receptor-effector coupling in transmembrane signaling. Our studies also indicate that homeoviscous adaptation per se is not responsible for the differences in pressure response.

WORK PLAN (next 12 months): The objectives for next year's work are to continue and complete characterization of GTPase activity at elevated pressure, examine the effects of hydrostatic pressure on pertussis toxin-catalyzed ADP-ribosylation, and other experiments focusing on the differences in the G protein pools of the two Sebastolobus species.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. J.F. Siebenaller and T. F. Murray (1990) A<sub>1</sub> adenosine receptor modulation of adenylyl cyclase of a deep-living teleost fish, Antimora rostrata. Biological Bulletin 178: 65-73.
2. J.F. Siebenaller (1991) Pressure as an environmental variable: Magnitude and mechanisms of perturbation. In: Biochemistry and Molecular Biology of Fishes, Vol. 1, P.W. Hochachka and T.P. Mommsen (editors), Elsevier Press, in press.
3. J.F. Siebenaller, A.F. Hagar and T.F. Murray (1991) The effects of hydrostatic pressure on A<sub>1</sub> adenosine receptor signal transduction in brain membranes of two congeneric marine fishes. Journal of Experimental Biology, in press.

## ANNUAL PROGRESS REPORT

Grant #: N00014-88-K-0462

R&T CODE: 4415804

PRINCIPLE INVESTIGATOR: Anne Walter

INSTITUTION: Wright State University

GRANT TITLE: Effect of Pressure on Membrane Fusion: A Means to Identify Molecular Mechanisms.

REPORTING PERIOD: 1 July 1989 - 30 June 1991

AWARD PERIOD: 1 July 1988 - 30 June 1991 (decision pending on a 1 year no cost extension to 30 June 1992)

OBJECTIVE: To discriminate among proposed lipid intermediates for the process of membrane-membrane fusion by determining fusion rates of several lipid-fusagen systems as a function of temperature and pressure.

ACCOMPLISHMENTS (last 24 months): (1) We determined that **divalent-cation-induced fusion of PS-vesicles was sensitive to the choice of acyl chain**: POPS is more recalcitrant to fusion than the mixed chain analog bovine brain PS. On the basis of these results, we have sacrificed the sharper T<sub>c</sub> of POPS for bovine brain PS which appears to give real fusion. (2) We have **separated aggregation and fusion rate kinetics** using 90° scatter and lipid mixing (NBD-PE and Rho-PE labeled vesicles fuse to unlabeled vesicles and the relief of energy transfer is observed). By collecting data under **stopped-flow conditions**, we have shown that it is feasible to determine the **initial rates** (dimer formation) of each process and clearly separated these from later events associated with cochleate structure formation. Clearly identifying the region of dimer formation from two independent parameters (aggregation and fluorescence changes associated with fusion) was essential as the models used to calculate these rates were found by us to involve a numerical solution that is not valid at very early times. The energy transfer assay is internally consistent and no artifacts appear to complicate the data analysis. (3) The **temperature dependence** of aggregation rates appears to be close to that expected for the change in water viscosity whereas, fusion (lipid mixing) is very temperature dependent, with a threshold above, but near, T<sub>c</sub>. (4) We showed that the collisional quencher DPX (*p*-xylene-bis-(pyridinium bromide)), frequently used for contents mixing assays, **both decreased T<sub>c</sub> and increased the vesicle fusion rate** without affecting the aggregation rate. This result explained a previous problem, i.e., the fusion rates appeared to depend on the ratio of fluorescently labeled and DPX containing vesicles. We hypothesize that DPX is binding to the PS surface electrostatically: the increase in rate may be due directly to the change in T<sub>c</sub> (certainly near T<sub>c</sub>), due to neutralization of charges, or due to some other unidentified factor. (5) The effects of two divalent cations on aggregation and fusion were compared under fusion-rate limiting conditions. Barium and calcium (3 mM) both induce aggregation of and lipid mixing between bovine brain PS vesicles that increases with

temperature (10 - 50°C). T<sub>c</sub> for Ba<sup>2+</sup>/PS mixtures is about 30°C, but no particular break in the fusion rate was observed at or around this temperature. However, the temperature dependence for fusion induced by Ca<sup>2+</sup> is much steeper than that induced by Ba<sup>2+</sup>. The apparent higher activation energy may indicate some fundamental difference as to how the two ions function. (6) Preliminary experiments have been performed to compare the effects of adding different types of 'acyl chains' to the bilayer by comparing hexadecane (5 to 10 mol%) and dioleoylglycerol (DOG) (3mol%) effects on T<sub>c</sub>, aggregation and fusion rates of bovine brain PS vesicles. At present, it appears that hexadecane has no effect on any of these parameters except to broaden and slightly (not significantly) raise T<sub>c</sub>. DOG, however, has relatively little effect on T<sub>c</sub> but does appear to increase fusion rates significantly. This latter experimental result must be repeated to be believed.

WORK PLAN: (next 12 months): The goals for the next year are to submit the above work for publication, to apply the new assay conditions to actual fusion problems, and make the high pressure stopped flow useable for fluorescence determinations. Two manuscripts are currently in preparation based on the results described above. We will complete the work begun with PS/alkane and PS/DAG systems (item 6 above) to confirm and identify the basis of the apparently dramatic effect of DOG. Other DAG's will be tested, the temperature dependence will be determined and the effect of pressure examined. The high pressure stopped flow is in place, but needs to be optimized for fluorescence measurements: getting this instrumentation functional is our major priority and initial experiments will be done to follow leakage of vesicle contents since fluorescence should not be limiting for these assays, whereas it may be for lipid mixing assays. The effect of barium and calcium will be examined at pressure in an attempt to identify the source of the vary different temperature dependencies of PS vesicle fusion induced by these two ions. If warranted, similar experiments will be performed using monomethyl-phosphatidylethanolamine.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Walter, A., J.L. Banschbach and D.P. Siegel. 1991. Measuring vesicle fusion rates using fluorescence assays: some considerations revisited. Abstract presented at the 1991 Biophysical Society Meeting. Biophys. J. 59:128a.
2. Walter, A., S. Lesieur, R. Blumenthal, and M. Ollivon. 1991. Size characterization of liposomes by HPLC. invited chapter for LIPOSOME TECHNOLOGY 2nd edition. in the press.

**SENSORY BIOPHYSICS I**

**CORE PROGRAM**

**SCIENTIFIC OFFICER: Dr. Igor Vodyanoy**

**BEGAN: OCTOBER 1 1988**

**PROGRAM OBJECTIVE: TO UNDERSTAND THE PHYSICAL-CHEMICAL  
MECHANISMS OF MEMBRANE AND CELLULAR EVENTS CONCERNED WITH  
THE SENSING OF PHYSICAL FORCES.**

**NAVY OBJECTIVE: TO EXPLOIT THE BIOLOGICAL PRINCIPLES OF  
SENSING FOR THE DEVELOPMENT OF MILITARILY USEFUL DEVICES AND  
TO IMPROVE PERSONNEL PERFORMANCE.**

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1700

R&T CODE: 4414905

PRINCIPAL INVESTIGATOR: Guenter Albrecht-Buehler

INSTITUTION: Northwestern University Medical School

GRANT TITLE: Cellular detection of infrared sources

REPORTING PERIOD: 2 June 1990 - 3 June 1991

AWARD PERIOD: 1 March 1989 - 29 February 1992

OBJECTIVE: The development of appropriate assays to test whether individual mammalian tissue cells are capable of detecting the location of microscopic infrared sources in their environment and directing their migration towards them.

ACCOMPLISHMENTS: We have developed two assays to approach the objective. In one assay we tested whether infrared irradiation of a microscopic spot in the tail region of a migrating 3T3 fibroblast can cause the cell to reverse the direction of its migration. The second assay tested whether such a cell was able to detect and reach over to a microscopic latex particle located 10 - 100  $\mu\text{m}$  away from the cell if the particle scattered the light from a beam of infrared light. We found that 25% of the tested cells expressed both these remarkable abilities. The peak response was found in the range of wavelengths between 800 and 900 nm. The light intensity had to alternate at a frequency of about 30-60 Hertz.

SIGNIFICANCE: Conceptually, the results suggest that mammalian tissue cells may have a signal-integration and motility-coordinating mechanism that may be compared to the nervous control mechanisms of multicellular organisms. Practically, they may suggest techniques to attract or repel migrating tissue cells by appropriate infrared irradiations that may be useful in the treatment of wounds and malignant tumors.

WORK PLAN (next 12 months): The most urgent next step is the localization of the putative 'control- and integration-center' if, indeed, it is localized. We have hypothesized earlier that the centrosphere would be a promising candidate for such a control and integration center. Therefore, we will focus on two experimental plans.

a. Local illumination of the centrosphere with intermittent infrared light between 800-900 nm to test whether the such 'blinded' cells alter their motile behavior. This series of experiments together with its controls will involve a large number of single live cell

observations.

b. Illumination of whole dishes of cells at once with the active wavelengths and illumination protocol. Several years ago, we developed a new method to allow large numbers of migrating tissue cells in culture to record their movements by their 'phagokinetic tracks'. In this assay the cells migrate over a carpet of colloidal gold particles on the substrate and leave particle-free tracks as a record of their locomotion. We plan to combine this assay with the infrared illumination of whole dishes to examine the effects of intermittent infrared illumination on the phagokinetic track patterns of migrating 3T3 cells. In this way we hope to develop faster methods to examine the phenomenon.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1669

R&T CODE: 4413042

PRINCIPAL INVESTIGATOR: Eugene M Goodman

CONTRACTOR: University of Wisconsin-Parkside

GRANT TITLE: Weak Electromagnetic Field Effects on Gene Expression in *E. coli*

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 June 1990 - 31 May 1993

OBJECTIVE: Previous experiments have shown that exposing *Escherichia coli* to a pulsed magnetic field (PMF) increased and/or decreased a significant number of proteins. The current program is directed to determining if the altered proteins profiles occurred as a result of changes in transcription, translation or both processes. In addition we are also examining the possibility that the supercoiled state of DNA has been altered as a result of PMF exposure.

ACCOMPLISHMENTS: During the past year we applied an *in vitro* transcription/translation system to determine if electromagnetic field (EMF) effects can be induced in a cell-free system, and to address the question of EMF effects on translation. Experiments were performed using an *E. coli* cell-free (S-30) cytoplasmic extract; once isolated, the extract was optimized for various cations using a plasmid containing 8 galactosidase as a reporter. The S-30 extract was used to assess the effect of weak fields on translation by adding a plasmid containing an  $\alpha$  polymerase insert to an S-30 mix containing <sup>35</sup>S methionine. One set of 4 samples was placed in the non-exposed control environment and the other in a PMF environment. Another set of negative controls (no plasmid) was placed in both environments. The mix was exposed to PMF's for one hour whereupon the newly labelled protein is precipitated and the incorporated label was determined. The data obtained to date strongly suggest that exposure to PMF's enhanced (~20%-30%) the incorporation of <sup>35</sup>S methionine into acid precipitable protein relative to non-exposed control extracts. We are currently attempting to decrease the variance in the experiment.

A second set of experiments has been recently initiated to examine the question of whether the changes in protein profiles was associated with enhanced transcription. In initial experiments *E. coli* were grown to late log phase in either a control or electromagnetic field; at the end of the growth phase total RNA's were isolated from each culture. The RNA was blotted to a nitrocellulose membrane and probed using an 850 bp segment from the 5' end of  $\alpha$  polymerase. At this

time, we have isolated and labelled the probe and blotted the RNA's; we are just beginning to perform the slot-blot hybridizations.

In the final set of experiments we are subjecting bacteria containing a plasmid (pluc) to both a non-exposed control environment and a PMF's. The plasmids are isolated, purified and separated on chloroquine gels. The degree of supercoiling is assessed and visualized with ethidium bromide under uv light. At this time, we are unable to conclude the extent to which supercoiling has been affected by EMF exposure.

Significance: The application of a cell free *in vitro* transcription/translation system will allow us to experimentally examine the often theorized requirement for an intact membrane to transduce an electromagnetic field effect. In fact, our preliminary data suggest this is not the case. In addition, the experimental design of this program should to a significant degree address the question of the mechanism of interaction between weak electromagnetic fields and living systems.

WORK PLAN (next) 12 months): During the next 12 months we intend to fine tune the cell free S-30 experiments to unequivocally show that EMF's are effective in an EMF environment. We also intend to pursue experiments designed to establish whether transcription and/or translation are directly affected by EMF exposure. Finally, work on the relationship between supercoiling and EMF exposure will be continued.

PUBLICATIONS AND REPORTS: (last 12 months)

Goodman, E.M., Greenebaum, B., and M.T. Marron (1991). *Altered Protein Synthesis in a Cell Free System Exposed to a Pulsed Electromagnetic Field*. Ann. Meeting Bioelectromagnetics Soc. B-2-2

Smith, O., Goodman, E.M., Greenebaum, B., and Tipnis, P. (1991) *An increase in the negative surface charge of U937 cells exposed to pulsed magnetic fields*. (in press, Bioelectromagnetics)

*Effects of pulsed magnetic fields on proteins in E. coli* (manuscript in preparation).



# ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1318

R&T CODE: 4414047

PRINCIPAL INVESTIGATOR: Reba Goodman

INSTITUTION: Columbia University

GRANT TITLE: Cellular Response to Exogenous Hybridization

REPORTING PERIOD: 1 July 1990 - 3 June 1991

AWARD PERIOD: 1 October 1989 - 30 September 1991

OBJECTIVE: To determine whether transcriptional patterns are affected when dipteran salivary gland cells are exposed to extremely low frequency (elf) electromagnetic (EM) fields, and if so, to identify the following: (a) the specific gene regions involved (using published maps), (b) the time frame within which the transcriptional changes occur and their duration, (c) when accommodation to the EM field occurs, and, eventually (d) the initiating factor responsible for the affects as well as the signal component responsible for the alterations in gene expression.

ACCOMPLISHMENTS: We have used transcription autoradiography to identify transcriptional activity in defined regions of *Drosophila* salivary gland chromosomes. Following a twenty minute exposure to elf EM fields, we have shown that the transcriptional activity of some transcripts was affected. Two chromosomal regions (87AD and 93BD) showed transcriptional activity following either heat shock or EM field exposure. Chromosomes from cells exposed to EM fields showed transcriptional activity in at least 13 additional regions of chromosome 3R as compared with the unexposed control preparations. Six chromosomal regions showed a signal specific response. Many of the chromosome regions affected by elf EM fields contain loci related to growth; e.g. tubulin and actin. Some of the transcriptional increases are reflected in an increase in polypeptide synthesis as well as in the number of polypeptides resolved in cells exposed to electromagnetic fields. A detailed analysis of chromosome 3R is in press (Bioelectromagnetics, 1991). Analyses of chromosomes 3L and X are in preparation for publication.

SIGNIFICANCE: *Drosophila* salivary gland cells are a useful initial model for determining the nature of the regulatory processes affected by cellular exposure to elf EM fields. These cells can be used for tentative identification of gene loci affected by short term exposure EM fields. Information derived from this approach is ultimately tested in human cells. It is expected that this approach will allow us to estimate the total number of genes affected in cells exposed to EM fields, and to determine whether the affected transcript have regulatory sequences in common. Should this prove to be the case, it would support our current hypothesis that a specific subset of genes is sensitive to EM field exposure.

WORK PLAN (next 12 months): The objective of the next 12 months is to complete the mapping and chromosomal regions affected by short exposure (5 to 60 minutes) to elf EM fields. transcription radiography, *in situ* hybridization together with detailed chromosome maps and gene data banks will enable us to identify chromosomal regions and the genes within these regions that show transcriptional augmentation or suppression as compared with the unexposed control cells. We will attempt to determine how many total genes are affected and if regulatory sequences common to the affected transcripts are present. Alterations in polypeptide synthesis in cells exposed to EM fields continue to be analyzed by one-

and two dimensional gel electrophoresis. Augmentation and suppression of transcription in specific chromosomal regions will be correlated with exposure conditions; e.g., variations in frequency (5 to 150 Hz and higher) and varying field strengths ( $5.7 \times 10^{-3}$  to 5.7 Gauss).

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Goodman, R., D. Weisbrot, A. Uluc, and A. Henderson, (1991) Transcription in Drosophila melanogaster salivary gland cells is altered following exposure to low frequency electromagnetic fields: Analysis of chromosome 3R. Bioelectromagnetics, in press.
2. Wei, L.-X., R. Goodman, and A. Henderson, (1990) Changes in levels of c-myc and histone H2B following exposure of cells to low-frequency sinusoidal electromagnetic fields: evidence for a window effect. Bioelectromagnetics 11:269-272.
3. Goodman, R. and A. Henderson, (1990) Exposure of cells to extremely low-frequency electromagnetic fields: Relationship to malignancy? Cancer Cells 2: 355-359.
4. Goodman, R. and A. Henderson (1991) Transcription and translation in cells exposed to extremely low frequency electromagnetic fields. Bioelectrochem. Bioenerget, in press.
5. Goodman, R., L.-X. Wei, J. Bumann, and A. Henderson (1991) Exposure of human cells to electromagnetic fields: Effect on transcription. Submitted to FASEB Journal.
6. Goodman, R., L.-X. Wei, J-C Xu, and A. Henderson (1990) Transcriptional changes in cells exposed to elf electromagnetic fields. abstract presented at the 10th Ann. Bioelectrical Chemical and Growth Soc. Philadelphia, PA.
7. Goodman, R., L.-X. Wei, J. Bumann, and A. henderson (1990) Relationship of field strength and time to increased levels of transcripts induced by elf EM fields, abstract presented at the Ann. Review of Research on Biological Effects of 50 and 60 HZ Electric and Magnetic Fields -DOE/EPRI, Denver, A38.
8. Wei, L.-X., R. Goodman, J. Bumann, and A. Henderson (1990) Transcriptional changes in cells exposed to low frequency electromagnetic fields. abstract presented at the 30th Annual American Soc. Cell Biology, San Diego, 510A.
9. Wei, L.-X., R. Goodman, J-C Xu and A. henderson (1991) Transcription is affected by low frequency electromagnetic fields, abstract presented at FASEB, Washington, D.C.
10. Goodman, R., L.-X. Wei, and A. Henderson (1991) Relationship of field strength and time of induction to increased transcript by elf EM fields. abstract presented at RCMI Symposium, Atlanta.
11. Goodman, R., J. Bumann and A. Henderson (1991) Defining signal parameters which induce transcriptional/translational alterations in HL-60 cells. abstract presented at 13th Ann. Bioelectromagnetics Soc. Salt Lake City, in press.
12. Goodman, R. and A. Henderson (1991) Transcriptional changes in cells exposed to extremely low frequency electromagnetic fields, FASEB, Atlanta, in press.

# ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1266

R&T CODE: 4414805

PRINCIPAL INVESTIGATOR: Ann S. Henderson

INSTITUTION: Hunter College-City University of New York

GRANT TITLE: Exposure of Human Cells to Electromagnetic Fields

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 January 1990 - 31 December 1991

OBJECTIVE: To determine if transcription (and resulting translation) is altered in human cells exposed to extremely low frequency (elf) electromagnetic (EM) fields; if transcription is altered, to determine the characteristics of the EM fields and cells that can be related to the effect.

ACCOMPLISHMENTS: Our experiments show a pronounced measurable response observed as transcript increase in the human cultured cell line, HL-60, with associated changes in protein synthesis. The major findings relative to transcriptional changes are fourfold: (1) transcript changes in human cells correlate with previous findings in *Drosophila* salivary gland cells; (2) the frequency of the signal in the range from 15 to 150 Hz results in a "window" at 45 Hz; (3) changing the signal strength in four log increments (starting from about twice laboratory background levels) both amplitude and time-dependent windows, and (4) genes not usually expressed in HL-60 are unaffected by exposure to elf EMFs. Changes in the overall protein synthetic pattern have also been observed following exposure of HL-60 cells to 60 Hz signals.

SIGNIFICANCE: A major question is how (or if) elf EMFs initiate a series of diverse biologic events within cells. Our research has approached the question of the conditions under which transcription within cells is affected.

WORK PLAN (next 12 months): Our present aim is to determine how elf non-ionizing radiation affects human cells. Specifically, are there molecular features of the affected cell that can be directly related to signal parameters such as frequency, B-field (or other signal parameters) and exposure time? **Our aims are as follows:** (1) to compare results using different signals; which parameters are critical to achieving a response on the part of the cell; (2) to determine how soon an effect can be identified following exposure of cells to elf EM fields; what is the time course of the response; is it the same for each transcript measured; (3) to determine how long the biologic affect exist; is there cellular adaptation to the presence of the signal; (4) to determine the consistency of results using different molecular analysis methods. The broad goals are to seek biologic parameters which are critical. For example, is transcription affected at the nuclear level, or are our results detecting stored cytoplasmic mRNA species? Another goal is to delineate how many transcripts are affected by elf EM fields and if these represent special subsets of genes.

## PUBLICATIONS AND ABSTRACTS (last 12 months):

Goodman, R., D. Weisbrot, A. Uluc and Ann Henderson, 1990.

Transcriptional responses in *Drosophila melanogaster* salivary gland cells are altered following exposure to low frequency electromagnetic fields. Bioelectromagnetics, in press.

Wei, L.-R., R. Goodman and A. Henderson, 1990. Changes in levels of c-myc and histone H2B following exposure of cells to low frequency sinusoidal signals: Evidence for window effect. Bioelectromagnetics

11:269-272.

Goodman, R. and A. Henderson, 1990. Transcription in cells exposed to extremely low frequency electromagnetic fields: a review.

Bioelectrochem. Bioenerget, in press.

Goodman, R. and A. Henderson, 1990. Exposure of cells to extremely low frequency electromagnetic fields; relationship to malignancy? Cancer Cells 2:355-359.

Goodman, R., J. Bumann, L.-X. Wei, J-C Xu and A. Shirley-Henderson, 1991. Transcriptional changes in cells exposed to extremely low frequency electromagnetic fields. BRAGS 10th Anniversary Volume, in press.

Goodman, R., L-X wei, J-C Xu and Ann S. Henderson, 1990, Exposure of human cells to electromagnetic fields: The amplitude changes affect the level of transcripts. J. Bioelect., in press.

#### Abstracts:

Goodman, R., L-X Wei, J-C Xu and A. henderson, 1990. Quantitative changes in transcripts result from varying signal amplitude. Trans. 12th Annual Bioelectromagnetics Soc., San Antonio.

Goodman, R., L-X Wei, J-C Xu and A. Shirley-Henderson, 1990. Transcriptional changes in cells exposed to elf electromagnetic fields. 10th Annual Bioelectromagnetics Soc., Philadelphia.

Goodman, R, L-X Wei, J. Bumann, and Ann S. Henderson, 1990. Relationship of field strength and time to increased levels of transcripts induced by elf EM fields. The Annual Review of Research on Biological Affects of 50 and 60 Hz Electric and Magnetic Fields DOE/EPRI, Denver, A38.

Wei, L-X, Goddman, R., J. Bumann and A. Henderson, 1990.

Transcriptional changes in cell exposed to extremely low frequency electromagnetic fields. 30th Annual Meeting American Soc. Cell Biology, San Francisco, p.105A.

Goodman, R., L-X Wei, J. and A. Henderson, 1991. Relationships of fields strength and time to induction of increased transcripts by elf EM fields. RCMI Symposium, Atlanta.

Goodman, R., J. Bumann and A. Shirley-Henderson, 1991. Defining signal parameters which induce transcriptional/translational alterations in HL-60 cells. Bioelectromagnetics Soc., Salt Lake City, in press.

Goodman, R. and A. Henderson, 1991. Transcriptional changes in cells exposed to extremely low frequency electromagnetic fields. FASEB, Atlanta, in press.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1962

R&T CODE 4414030

PRINCIPAL INVESTIGATOR: Dr. Leslie M. Loew

INSTITUTION: University of Connecticut Health Center

GRANT TITLE: The Optical Patch Clamp. Stage 1: Second Harmonic Generation with Potentiometric Membrane Probes

REPORTING PERIOD: 1 June 1990 - 31 May 1991 (12 months)

AWARD PERIOD: 1 June 1990 - 31 May 1992

OBJECTIVE: To prepare and characterize a series of chiral potentiometric dyes based on the aminonaphthylethenylpyridinium chromophore; to test the non-linear optical properties of these dyes in monolayer assemblies; to determine if second harmonic generation by these dyes is sensitive to membrane potential.

ACCOMPLISHMENTS (last 12 months): The synthesis of 3 chiral dyes were achieved using the alkylation of the pyridine nitrogen in 1-(6-dialkylaminonaphth-2-yl)-2-(pyridin-4-yl)ethene. The alkylating agents were optically active and did not racemize during the reaction. The spectral properties of these new compounds were determined; as expected, they have absorbance and fluorescence spectra very similar to other members of the ANEP family of dyes which were developed in this laboratory. The voltage and wavelength dependence of their spectral responses to membrane potential pulses were also determined using a voltage clamped spherical bilayer apparatus. In one case, the response was short-lived, corresponding possibly to flip-flop of the probe across the lipid bilayer.

SIGNIFICANCE: These newly synthesized dyes, which are amphiphilic and contain both intrinsic asymmetry and a chromophore which undergoes a large charge redistribution upon excitation, should be ideally suited for production of molecular assemblies with large non-linear optical properties.

WORK PLAN (next 12 months): Our primary objective will be to determine whether the new dyes do indeed display second harmonic generation. We will use the Nd-Yag laser and Langmuir-Blodgett monolayer systems in Dr. Aaron Lewis' laboratory at the Hebrew University to test these dyes. Since other achiral members of this class of dyes are efficient frequency doublers, we are confident that these new dyes will work. We will also set up a voltage clamped spherical bilayer apparatus there; this will permit us to see if we can measure second harmonic signals when the dye is diluted into a lipid bilayer. Finally we hope to test the idea that the efficiency of second harmonic generation by these dyes is dependent on membrane potential.

PUBLICATIONS AND ABSTRACTS (last 12 months): none

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1715

R&T CODE: 4414708

PRINCIPAL INVESTIGATOR: Dr. Arthur E. Sowers

INSTITUTION: Jerome H. Holland Laboratory (American Red Cross)

GRANT TITLE: Post-Fusion Membrane Reorganization

REPORTING PERIOD: 1 February 1991 - 3 June 1991

AWARD PERIOD: 1 February 1991 - 31 January 1991

OBJECTIVE: Determine how viscosity, temperature, perpendicular force, and fusogen (electric pulse) affect the membrane fusion product final stable ultrastructure. Attempt to capture pre-stale fusion product ultrastructure by both phase contrast light microscopy and electron microscopy. Determine if membrane area outside of the contact zone comes into the fusion zone. Determine if biological differences between membranes of human and rabbit erythrocytes have effects on product fusion formation.

ACCOMPLISHMENTS (last 12 months): Experimental work was concluded on the interference effect between chemical substances added to the aqueous media (needed before chemical viscosity modifiers are added for part of the above stated objective) and fusogenic electric pulse. Attempts to induce membrane fusion by membrane-membrane contact brought about by dielectrophoresis using chemicals known to be fusogenic were negative. The presence at very low concentrations of soluble macromolecular species interferes with electrofusion yield; fusion yields are increased up to 200-300% or decreased to near zero depending on chemical species and concentration range. The design and construction of a large chamber for obtaining electrofusion products in large quantities is progressing.

SIGNIFICANCE: Absence of membrane fusion in erythrocyte ghosts induced by use of chemical fusogens and dielectrophoresis means suggests that all chemical fusogens may require participation of an active, intact, and viable cytoplasm. This is significant for a better understanding of how chemical fusogens work. The fusogenic effect may be a secondary rather than a primary effect. The strong interference effect is both surprising (in terms of its magnitude for such low concentrations) and unexpected (no theory or hypothesis predicts it). Regardless of whether the electrofusion mechanism involves electropores, the presence of macromolecular species at very low concentrations is clearly modulating some critical step in the mechanism.

WORK PLAN (next 12 months): We plan to carry out the proposed experiments as described (see objectives, above).

PUBLICATIONS AND ABSTRACTS (last 12 months):

Ia. Publications in Peer-Reviewed Journals:

- Dimitrov, D.S., and Sowers, A.E. (1990) A Delay in Membrane Fusion: Lag Times Observed by Fluorescence Microscopy of Individual Fusion Events. *Biochemistry* 29, 8337-8344.
- Chernomordik, L.V., and Sowers, A.E. (submitted revised version) Evidence that the Spectrin Network and a Non-Osmotic Force Controls the Fusion Product Morphology in Electrofused Erythrocyte Ghosts. *Biophys. J.*
- Abidor, I.G., and Sowers, A.E. (submitted) Kinetics and Mechanism of Cell Membrane Electrofusion. *Biophys. J.*

Ib. Books and Book Chapters:

- Chang, D.C., Chassy, B.M., Saunders, J.A., Sowers, A.E., eds. (submitted) Handbook of Electroporation and Electrofusion, (Academic Press).
- Sowers, A.E. (submitted) Membrane Electrofusion: A New Paradigm for the Study of Membrane Fusion Mechanisms. In: Methods in Enzymology (N. Duzgunes, ed.)
- Sowers, A.E. (submitted) Mechanisms of Electroporation and Electrofusion. In: Handbook of Electroporation and Electrofusion (D.C. Chang, B.M. Chassy, J.A. Saunders, and A.E. Sowers, eds.) Academic Press, San Diego.
- Chang, D.E., Saunders, J.A., Chassy, B.M., and Sowers, A.E. (submitted) An Overview of Electroporation and Electrofusion. In: Handbook of Electroporation and Electrofusion (D.C. Chang, B.M. Chassy, J.A. Saunders, and A.E. Sowers, eds.) Academic Press, San Diego.
- Chassy, B.M., Saunders, J.A., and Sowers, A.E., (submitted) Pulse Generators for Electrofusion and Electroporation. In: Handbook of Electroporation and Electrofusion (D.C. Chang, B.M. Chassy, J.A. Saunders, and A.E. Sowers, eds.) Academic Press.
- Sowers, A.E. (submitted) The Role of Both Post-Fusion Lumen Expansion and Membrane Fusion in the Entry of Cells by Enveloped Viruses. In: Advances in Membrane Fluidity series, Vol 6 - Membrane Interactions of Human Immunodeficiency Virus (R.C. Aloia, C.C. Curtain, and L.M. Gordon, eds) John Wiley and Sons, Inc.

II. Published Abstracts:

- Rols, M.-P., and Sowers, A.E. (1991) Membrane Perturbing Agents, Including Fluidizers, Significantly Affect Electrofusion Yields. *Biophys. J.* 59, 205a.
- Chernomordik, L.V., and Sowers, A.E. (1991) Evidence that Spectrin Restrains the Expansion of a Fusion Zone if it is Created Between Two Erythrocyte Ghosts in Contact and Even if the Spectrin is Intact in Only One of the Two Ghost Membranes. *Biophys. J.* 59, 209a.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-88-J-1220

R&T CODE: 441K703

PRINCIPAL INVESTIGATOR: Dr. Tian Y. Tsong

INSTITUTION: University of Minnesota

GRANT TITLE: Effect of Electric Fields on Membrane Bound Na,K-ATPase

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 January 1990 - 31 December 1993

OBJECTIVE: To understand how cells can receive, decipher and respond to electric signals; to develop theory of electro-conformational coupling to explain electric activation of membrane enzymes and transporters.

ACCOMPLISHMENTS: To demonstrate that Na,K-ATPase is not the only enzyme which can respond to an electric stimulation we have performed electric stimulation on Ca-ATPase of human erythrocyte,  $F_0F_1$ -ATPase of beef heart submitochondrial particles, and purified, solubilized ECTO-ATPase from chicken oviduct. In all these cases, enzymes responded to the applied oscillating electric fields. Each enzyme showed a frequency and an amplitude windows for optimal stimulation.

We have also extended the theory of the electroconformational coupling to cover five types of transport systems: 1) a transporter with gating charges and a neutral ligand; 2) a transporter without gating charges and a charged ligand; 3) a transporter with gating charges but neutralized by the charges of the ligand, 4) a transporter and its complex with the ligand having gating charges of the opposite signs; and 5) a transporter with gating charges with the same sign as the charges of the ligand. These five types exhaust all the electrically active transport systems. Frequency, amplitude and ligand concentration windows for electric activation have been derived and expressed in terms of the rate constants for a Michaelis-Menten enzyme. Energy transduction efficiency for each type has also been derived.

SIGNIFICANCE: All enzymes tested can either recognize or respond to specific electric signals. Electric activation may explain how cells and organisms can interact with the electromagnetic field and is likely a fundamental process for cellular energy and signal transduction.

WORK PLAN (next 12 months): Electroconformational coupling can explain the results of Na,K-ATPase and Ca-ATPase. But, it may not explain the results of  $F_0F_1$ -ATPase and ECTO-ATPase because electric fields experienced by these enzymes would be too small. Modification of the theory or development of new theory will be attempted. Purified Na,K-ATPase and ECTO-ATPase will be reconstituted into lipid vesicles for electric stimulation. This is necessary to prove that an ATPase alone can transduce electric energy. So far, we have not experimentally observed a ligand concentration window. Search of such a window will be attempted.

### PUBLICATIONS AND REPORTS (7 abstracts not included):

1. Xie, T.-D. and Tsong, T.Y. (1990). Study of mechanisms of electric field induced DNA transfection II: Transfection by low amplitude low frequency alternating electric fields. Biophys. J. 58: 897-903.

2. Markin, V.S., Tsong, T.Y., Astumian, R.D. and Robertson, B. (1990). Energy transduction between a concentration gradient and an alternating electric field. J. Chem. Phys. 93:5062-5066.



3. Marszalek, P., Liu, D.-S. and Tsong, T.Y. (1990). Schwan Equation and transmembrane potential induced by oscillating electric field. *Biophys. J.* 58: 1053-1058.
4. Tomita, M. and Tsong, T.Y. (1990). Selective production of hydridoma cells. *Biochim. Biophys. Acta* 1055:199-206.
5. Tsong, T.Y. (1990). On electroporation of cell membranes and some related phenomena. *Bioelectrochem. Bioenerg.* 24:271-295.
6. Prudovsky, I.A. and Tsong, T.Y. (1991). Fusions of fibroblasts with differentiated and non-differentiated leukemia cells results in DNA replication block. *Developmental Biology* 144:232-239.
7. Marszalek, P., Zielinski, J.J., Fikus, M. and Tsong, T.Y. (1991). Determination of electric parameters of cell membranes by a dielectrophoresis method. *Biophys. J.* 59:982-987.
8. Markin, V.S. and Tsong, T.Y. (1991). Frequency and concentration windows for the electric activation of a membrane active transport system. *Biophys. J.* June issue.
9. Markin, V.S. and Tsong, T.Y. (1991). Reversible mechanosensitive ion pumping as part of mechanoelectric transduction. *Biophys. J.* June issue.
10. Gimsa, J., Marszalek, J., Loewe, U. and Tsong, T.Y. (1991). Dielectrophoresis and electrorotation of *Neurospora* slime and murine Myeloma cells. *Biophys. J.* In press.
11. Popov, S.V., Svitkina, T.M., Margolis, L.B. and Tsong, T.Y. (1991). Mechanism of electric field induced cell membrane protrusion: The role of actin. *Biochim. Biophys. Acta.* In press.
12. Markin, V.S. and Tsong, T.Y. (1991). Electroconformational coupling for ion transport in an oscillating electric field: Rectification versus active pumping. *Bioelectrochem. Bioenerg.* In press.
13. Tsong, T.Y. (1991). Electroporation of cell membranes. *Biophys. J.* August issue.
14. Tsong, T.Y. and Tomita, M. (1991). Selective B-Lymphocyte-Myeloma cell fusion. *Methods in Enzymology.* In press.
15. Markin, V.S. and Tsong, T.Y. (1991). Thermodynamics of membrane transport in oscillating fields. *Modern aspects of Electrochemistry.* In press.
16. Tsong, T.Y. (1991). Electroconformational coupling: Enforced conformational oscillations of a membrane enzyme for energy transduction. "Biomembrane Electrochemistry", Blank, M. and Vodyanoy, I., Eds., ACS Book. In press.
17. Tsong, T.Y. (1991). Time sequence of molecular events in electroporation of cell membranes. "Handbook of Electroporation and Electrofusion", Chang, D.C., Sowers, A.E. et al. Eds. Academic Press. In press.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-91-J-1178

R&T CODE: 4414808

PRINCIPAL INVESTIGATOR: Dr. Harold H. Zakon

INSTITUTION: The University of Texas at Austin

GRANT TITLE: Signal Transduction in Electrorceptors

REPORTING PERIOD: 1 December 1990 - 1 July 1991

AWARD PERIOD: 1 December 1990 - 30 November 1992

OBJECTIVE: Weakly electric fish generate electric fields of a particular frequency around their bodies and detect those fields via a class of specialized sensory cells called electroreceptors. These cells are tuned to the fish's particular discharge frequency and their tuning may be shifted by steroid hormones. The aim of this proposal was to determine how the ion currents of the electroreceptor generate its frequency selectivity and extreme sensitivity to electric fields and how steroid hormones influence these ion currents.

ACCOMPLISHMENTS: In order to determine the ion currents of electroreceptors we wish to voltage clamp these cells. They are found in the skin encased in layers of cells connected with tight junctions and surrounded by connective tissue. We were able to disassociate skin with enzymatic treatment (collagenase, hyaluronidase, dispase), but were generally unable to determine whether disassociated cells were epidermal or sensory at the level of resolution afforded by our optics.

We tried two different approaches to gain access to the receptor cells. The first, was to cut slices of skin with a vibratome and to try to record from receptor cells in organs that had been sliced open. Unfortunately, we were never able to get consistent results with the slicing given the tough nature of the epidermis.

The second method has been more successful. We have gone back to our initial methods of cell dissociation but now we are able to direct out single organs from a patch of skin after cutting away the dermis. Four or more of these organs are placed in a dissociation medium and all the cells are dissociated. This greatly reduces the number of epidermal cells and increases our yield of receptor cells. In addition, a new procedure that we have tried is to selectively label the receptor cells (and support cells at the base of the organ) with the fluorescent lipophilic dye DiI, and this helps us to identify receptor cells after dissociation by visualizing the fluorescently-labeled cells.

SIGNIFICANCE: We have worked out a viable dissociation

technique for the electroreceptors.

WORK PLAN (next 12 months): We are now in a position where we will be able to begin recording from these cells with a patch electrode. We plan to begin this late summer or early fall once we improve the yield of receptor cells with our dissociation procedures. We will attempt to voltage clamp the dissociated receptor cells throughout the rest of the year.

**SENSORY BIOPHYSICS II**

**SIGNAL TRANSDUCTION IN VESTIBULAR RECEPTORS**

**ACCELERATED RESEARCH INITIATIVE**

**SCIENTIFIC OFFICER: Dr. Igor Vodyanoy**

**BEGAN: OCTOBER 1, 1988**

**ENDS: SEPTEMBER 30, 1992**

**PROGRAM OBJECTIVE: TO UNDERSTAND FUNDAMENTAL BIOPHYSICAL AND BIOCHEMICAL MECHANISMS GOVERNING THE OPERATION OF THE VESTIBULAR SYSTEM AND THEIR ROLE IN ADAPTATION TO MOTION.**

**NAVY OBJECTIVE: TO DEFINE OPTIMAL CONDITIONS FOR ENHANCING SENSITIVITY OF MAN-MACHINE INTERFACE AND TO ADDRESS RESEARCH ON DEGRADATION OF CREW PERFORMANCE RESULTING FROM SPATIAL DISORIENTATION AND MOTION SICKNESS.**

## ANNUAL PROGRESS REPORT

GRANT #: N00014-91-J-1027

R&T Code 441m805

PRINCIPAL INVESTIGATOR: Manning J. Correia Ph. D.

INSTITUTION: The University of Texas Medical Branch

GRANT TITLE: Studies of the Ionic Basis of Sensory Adaptation and Gain in Vestibular Hair Cell/Afferent Synapse

REPORTING PERIOD: 1 June 1990 - 1 June 1991

AWARD PERIOD: 1 November 1989 - 31 October 1992.

OBJECTIVE: To investigate the ionic currents in type II and type II hair cells, the vestibular transducers.

ACCOMPLISHMENTS (LAST 12 MONTHS): We (Lang and Correia, 1990), published a description of currents in pigeon type I hair cells and compared these currents to those we have previously reported for type II hair cells. We (Angelaki and Correia, 1991) completed a theoretical study of the resonance properties of pigeon type II hair cells. This analysis evaluated several electrical circuit models that have been put forth in the literature to quantitatively describe the ringing that occurs in the membrane voltage response to a current pulse input. We found that while previous models, that have been posited to describe ringing of neural structures, fit our hair cell ringing membrane potentials for low values of quality of resonance, these models were unable to fit the oscillating membrane potentials when the quality of resonance was around a level of 0.6 and above. We proposed a model that extended the contemporary models that incorporate a resistor, capacitor, and inductor element to include an admittance term that contains components to produce an underdamped response. We (Dickman and Correia, 1990) studied the role of efferent stimulation (discrete mechanical stimulation of the contralateral semicircular duct) on the gain of semicircular canal afferent responses and found that efferent stimulation increases and decreases the spontaneous firing rate of the pigeon vestibular primary afferent responses in the contralateral ear. Finally, during morphophysiological studies, we (Tu and Correia) have impaled 103 horizontal semicircular canal primary afferent preganglionic fibers. We recorded spontaneous discharge and the response to mechanical duct displacement at 0.20, 2.0 and 20 Hz. The resulting data are under analysis.

SIGNIFICANCE: Our patch clamp data suggest that type I hair cells may be less sensitive than type II hair cells. Our theoretical studies suggest that filtering occurs in type II and I hair cells and this filtering functions to increase the gain of the hair cell output at the range of frequencies near the resonant frequency. Finally our single unit extracellular data suggest that efferent fibers synapsing on type II hair cells and calyces of type I hair cells can modify the spontaneous transmitter discharge rate (as indicated by increased firing rate in primary afferents).

WORK PLAN (NEXT 12 MONTHS) As detailed in the original proposal, the last year of the grant will be devoted to studying the receptors for the neurotransmitters found in the type II hair cell membrane. These receptors will be the receptors for the efferent terminals that synapse on the type II hair cell. Based on previous work, acetylcholine (ACh) will be the leading candidate. Also since ACh has been observed in the interface between the calyx and type I hair cells and since we (Correia

and Lang, 1990) have reported an M-type (Ach modulated) potassium current in pigeon type I hair cells, Ach receptors will be studied in type I hair cells also. We will study these receptor sites by administering microliter doses to isolated hair cells using the concentration clamp technique (placing hair cells in a tube, Lang and Correia, 1989) and observing the whole cell membrane currents using the whole cell perforated patch clamp technique. Other candidate neurotransmitters that will be tested will be GABA, L-glutamate and L-aspartate. Using the concentration clamp method, a hair cell can be completely superfused in 15 msec. with no dilution of the superfusate. Dose response curves will be obtained.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Lang, D.G. and Correia, M.J. (1990) Membrane properties of type I semicircular canal hair cells isolated from the adult pigeon. Biophysical Abstr. 57, 129A.
2. Correia, M.J. and Lang, D.G. (1990) Electrophysiological differences between solitary type I and type II semicircular canal hair cells. Barany Society Abstr., 55.
3. Dickman, J.D. and Correia, M.J. (1990) Semicircular canal afferent response to efferent stimulation. Neurosci. Abst. 16, 734.
4. Correia, M.J. and Lang, D.G. (1990) An electrophysiological comparison of solitary type I and type II vestibular hair cells. Neurosci. Letters, 116: 106-111.
5. Correia, M. J. and Dickman, J.D. (1991) Peripheral Vestibular System, In Otolaryngology Vol. 1 Basic Sciences and Related Disciplines, 269-279.
6. Angelaki, D.E. and Correia, M.J. (1991) Models of membrane resonance in pigeon type II hair cells. Biol. Cybern. (in Press)

# ANNUAL PROGRESS REPORT

GRANT #: N00014-91-J-1159  
441m804

R&T CODE:

PRINCIPAL INVESTIGATOR: David P. Corey

INSTITUTION: Massachusetts General Hospital

GRANT TITLE: Adaptation and Regulation of the Transduction Mechanism in Vestibular Hair Cells

REPORTING PERIOD: 14 December 1990 - 1 June 1991

AWARD PERIOD: 14 December 1990 - 13 December 1992

OBJECTIVE: To determine whether adaptation in vestibular hair cells involves a mechanical adjustment within the stereocilia; to determine whether adaptation corresponds ultrastructurally to movement of the tip-link attachment point; to test whether a myosin-like protein could be the motor protein that drives adaptation.

ACCOMPLISHMENTS (last 6 months): The first of the three specific aims is going extremely well. Quantitative analysis of the positive and negative adaptation rates, at two membrane potentials, has led to a general theory of adaptation. When we add the measured spring constants of the stereocilia and the gating springs that open channels, the theory predicts a range of behavior that can be tested. We now have good quantitative agreement for magnitude of the shift in the sensitivity curve (the  $I[X]$  curve) with depolarization, for the timecourse of the shift, for the active movement of unrestrained bundles, and for the movement of bundles when the gating springs are cut. The quantitative correspondence between the adaptation process and the bundle movement makes it very likely that adaptation is in fact a mechanical process. Two other tests tend to confirm this: the voltage dependence of the two processes is essentially identical, and lowering calcium (which reduces adaptation) also reduces the active bundle movement. A manuscript has been written and is currently in the revision stage.

SIGNIFICANCE: We feel that this work settles the issue of how hair-cell adaptation works, at a biophysical level. Competitive theories (involving direct alteration of the channel protein) can be ruled out. For us, this represents a turning point, so that we now focus on the structures within stereocilia that constitute the motor.

WORK PLAN (next twelve months): The bulk of our effort over the next year will be directed at ultrastructural studies of adaptation. We now have good transmission and scanning electron microscopy of hair bundles in our own lab. (We had been collaborating previously.) We will be starting the large job of measuring positions of tip links and attachment points under different conditions of adaptation. This will involve several thousand micrographs, to get statistical significance. However, preliminary results are encouraging, and we have had no problems with methods, so I anticipate good progress.

PUBLICATIONS AND ABSTRACTS (last twelve months):

1. Shepherd, G.M.G., Corey, D.P., and Block, S.M. (1990) Actin cores of hair-cell stereocilia support myosin motility. Proc. Natl. Acad. Sci. USA 87:8627-8631.
2. Assad, J.A. and Corey, D.P. (1990) Adaptation in bullfrog saccular hair cells involves an active motor. Soc. Neurosci. Abstr. 16:1078 (442.1).
3. Corey, D.P., Tilney, L., Hudspeth, A.J. and Ashmore, J.F. (1990) SYMPOSIUM: Hair cells of the inner ear: Structure, transduction, and active motion. Soc. Neurosci. Abstr. 16:143.



## ANNUAL PROGRESS REPORT

GRANT #: N00014-88-K-0067

R&T CODE: 441m802

PRINCIPAL INVESTIGATOR: Dr. Dennis G. Drescher

INSTITUTION: Wayne State University

GRANT TITLE: Vestibular Ion Transport

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 November 1987 - 30 September 1991

OBJECTIVE: To identify sites of active ion transport and passive ionic conductance essential for vestibular transduction, and to identify mechanisms and molecular entities which may modulate ionic transduction currents of vestibular and related sensory cells.

ACCOMPLISHMENTS (last twelve months): We have found that: (1) presumptive mineralocorticoid receptor sites (Type I) that bind  $^3\text{H}$ -aldosterone are present in the cochlear lateral wall and ampulla of the guinea pig, and that the population of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase sites measured by  $^3\text{H}$ -ouabain binding in vitro can be up-modulated by exogenous aldosterone in these inner ear tissues; (2) tritiated quinuclidinyl benzilate ( $^3\text{H}$ -QNB) binding sites are concentrated at a morphological level corresponding to synaptic poles of vestibular hair cells, consistent with the presence of muscarinic acetylcholine receptors postsynaptic to the peripheral efferent boutons; (3) acetylcholinesterase is localized to efferent endings on saccular hair cells, and acetylcholine, measured by direct chemical assay, is present in the saccular nerve.

SIGNIFICANCE: (1) Our evidence suggests that vestibular epithelia, similar to kidney and other ion/fluid-transporting organs, are subject to functional regulation by aldosterone through modulation of the population of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase sites. (2) Our data support the idea that efferent boutons ending on vestibular sensory cells act by secreting a neurotransmitter that binds to muscarinic receptors. (3) Acetylcholine may be one neurotransmitter secreted by a population of peripheral vestibular efferents in the teleost, suggesting analogies to the mammalian vestibular sensory epithelium, where presumptive muscarinic cholinergic receptors have been detected.

WORK PLAN (next 4 months): Our goals for the remaining project period include the completion of ongoing studies employing grain density analysis of  $^3\text{H}$ -QNB binding, reflecting the presence of presumptive muscarinic acetylcholine

receptors in vestibular tissues. We will also continue to investigate the response of ATPase sites of inner-ear epithelia to stimulation by aldosterone, and quantitate and localize the ATPase sites by radiolabeled ouabain-binding, and also localize presumptive mineralocorticoid Type I receptor sites by radiolabeled aldosterone binding. Our most recent approach to describing molecular entities of vestibular ion transport is to amplify (by polymerase chain reaction) and sequence aldosterone receptor(s) in vestibular tissues of the guinea pig, in order to demonstrate presence and possible subtype(s) of this receptor specific to the mammalian vestibular periphery.

#### PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Kern, R.C., Kerr, T.P., and Getchell, T.V. (1991) Ultrastructural localization of  $\text{Na}^+/\text{K}^+$ -ATPase in rodent olfactory epithelium. *Brain Res.* 546: 8-17.
2. Khan, K.M., Hatfield, J.S., Drescher, M.J., and Drescher, D.G. (1991) The histochemical localization of acetylcholinesterase in the rainbow trout saccular macula by electron microscopy. *Neurosci. Lett.* In press.
3. Pitovski, D.Z., Drescher, D.G., Kerr, K.L., and Kerr, T.P. (1990) Effects of aldosterone on the binding of ouabain to inner-ear tissues. *Assoc. Res. Otolaryngol. Abstr.* 13: 303.
4. Drescher, M.J., Mandal, A., Gerber, M.E. and Drescher, D.G. (1991) Acetylcholine and dopamine identified in neural elements of the trout saccular macula. *Assoc. Res. Otolaryngol. Abstr.* 14: 37.
5. Khan K.M., Hatfield J.S. and Drescher D.G. (1991) Histochemical localization of acetylcholinesterase in the rainbow trout saccular macula. *Assoc. Res. Otolaryngol. Abstr.* 14: 37.
6. Pitovski, D.Z., Drescher, M.J. and Drescher, D.G. (1991) Mineralocorticoid (type I) receptors in the inner ear. *Soc. Neurosci. Abstr.* 17. Submitted.
7. Khan, K.M., Hatfield, J.S., Drescher, M.J. and Drescher, D.G. (1991) Electron microscopic localization of acetylcholinesterase activity in the rainbow trout inner ear. *Soc. Neurosci. Abstr.* 17. Submitted.
8. Kerr, T.P., and Drescher, D.G. (1991) Autoradiographic demonstration of presumptive muscarinic acetylcholine receptor sites in mammalian vestibular organs. In preparation.
9. Pitovski, D.Z., Drescher, M.J., Kerr, T.P., and Drescher, D.G. (1991) Aldosterone mediates increased binding of ouabain to inner-ear tissues. In preparation.

**SENSORY BIOPHYSICS III**

**ODORANT DISCRIMINATION PROGRAM**

**ACCELERATED RESEARCH INITIATIVE**

**SCIENTIFIC OFFICER: Dr. Igor Vodyanoy**

**BEGAN: OCTOBER 1, 1989**

**ENDS: SEPTEMBER 30, 1994**

**PROGRAM OBJECTIVE: TO CHARACTERIZE THE MOLECULAR MECHANISMS ASSOCIATED WITH THE DETECTION AND RECOGNITION OF ODORANT CHEMICALS BY OLFACTORY RECEPTOR NEURONS AND OTHER CELLS.**

**NAVY OBJECTIVE: TO PROVIDE INSIGHTS INTO THE PRIMARY OLFACTION CHEMICAL TRANSDUCTION MECHANISMS WHICH ULTIMATELY WILL LEAD TO CHEMICALLY-SENSITIVE NOVEL SENSOR DEVICES.**

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1566

R&T CODE: 441r002

PRINCIPAL INVESTIGATOR: Barry W. Ache

INSTITUTION: University of Florida

GRANT TITLE: Inhibition of Olfactory Receptor Cells: An Aquatic Model

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 March 1990 - 28 February 1993

OBJECTIVE: (1) To characterize the biophysical basis of odor-induced suppression in olfactory receptor cells, using lobster olfactory receptor cells, both in situ and in vitro, as an animal model. (2) To develop a compartmental model of the lobster olfactory receptor cell and to use this model to study signal transmission in the cell.

ACCOMPLISHMENTS (last 12 months): We completed defining the optimal conditions for culturing olfactory receptor cells in vitro (mss in preparation). We extensively characterized the odor sensitivity of the cells in vitro and ascertained that in terms of threshold, slope of the concentration-response function, rank order of stimulatory compounds, degree of tuning and the presence of opposing, odor-activated conductances, the response of the cultured cells matched that of lobster olfactory receptor cells in situ (mss in preparation). We used the cells in vitro to implicate bacterial toxin-insensitive G-proteins in transduction of both the inward and outward odor-activated currents (mss in preparation). We used the cells in situ to verify that activation of the suppressive conductance is sufficiently rapid to shape the output of the receptor cell (mss in preparation). In conjunction with Dr. Gordon Shepherd's lab (Yale Univ.), we developed a compartmental model of the lobster olfactory receptor cell and used this model to show that not only are the passive membrane properties of the cell sufficient to account for passive spread of the receptor potential, but that the electrotonic structure of the cell significantly improves signal recognition in the soma (mss submitted).

SIGNIFICANCE: The experimental and modeling data provide parallel lines of evidence that integration begins at the level of the receptor cell in the lobster olfactory pathway. The extent to which this organizational principal generalizes to other organisms awaits further work.

WORK PLAN (next 12 months): Combining the experimental advantages of both the in vitro and in situ preparations, we

plan to continue to define the transduction cascade associated with the suppressive conductance and to attempt to isolate and characterize the underlying channel. We also plan to extend the compartmental model of the lobster olfactory receptor cell to include the suppressive, as well as an excitatory, conductance in the ciliary arbor and use this model to determine the optimal ratio and spatial distribution of the two conductances for obtaining maximum diversity in the output of the cell.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Michel, W.C., McClintock, T.S., and Ache, B.W. 1991. Inhibition of lobster olfactory receptor cells by an odor-activated potassium conductance. J. Neurophysiol. 65:446-453.
2. Ache, B.W. Phylogeny of smell and taste. pp 3-18 In: Getchell, T. V. et al. (eds.) The Neural Basis of Smell and Taste. Raven Press, NY. (in press)
3. Pongracz, F., McClintock, T.S., Ache, B.W., and Shepherd, G.M. Signal transmission in lobster olfactory receptor cells: functional significance of electrotonic structure analyzed by a compartmental model. J. Neurosci. (Submitted).
4. Ache, B.W. Multiple transduction pathways activate different ionic conductances in lobster olfactory receptor cells. (Abstract: European Chemoreception Organization) Chem. Sens. (In press)
5. Fadool, D.A., Michel, W.C., and Ache, B.W., Odor and voltage dependent currents in cultured lobster olfactory neurons. (Abstract: Society for Neuroscience)
6. Michel, W.C. and Ache, B.W. Olfactory receptor cells output is shaped by summation of opposing, odor-activated conductances. (Abstract: Society for Neuroscience).
7. Pongracz, F., McClintock, T.S., Ache, B.W., and Shepherd, B.W. The electrotonic structure of lobster olfactory cells. (Abstract: Society for Neuroscience).
8. Fadool, D.A., Michel, W.C., and Ache, B.W. G-proteins mediate odor response in cultured lobster olfactory neurons. (Abstract: AChems) Chem. Sens. (In press)

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1503

R&T CODE: 441r009

PRINCIPAL INVESTIGATOR: Richard C. Bruch

INSTITUTION: Northwestern University

GRANT TITLE: Molecular Mechanisms of Olfactory Responses to Stimulus Mixtures.

REPORTING PERIOD: 15 March 1991 - 31 May 1991

AWARD PERIOD: 15 March 1990 - 14 February 1993

OBJECTIVE: To elucidate the molecular mechanisms underlying the responses of peripheral olfactory neurons to stimulus mixtures and to identify, at the molecular level, transduction proteins that mediate these responses.

ACCOMPLISHMENTS (last 1.5 months): Amino acid chemoreceptor in peripheral olfactory neurons of the channel catfish (*Ictalurus punctatus*) are linked to phosphoinositide hydrolysis by a GTP-binding regulatory protein (G-protein). However, the molecular nature of the G-protein linking the receptors to phospholipase C is unknown. We have found that pertussis toxin (PTX) markedly inhibits stimulus-dependent phosphoinositide hydrolysis in isolated olfactory cilia. For example, in the absence of PTX, L-alanine and L-arginine rapidly (within 15 sec) elicit large increases in IP<sub>3</sub> levels in the presence of exogenous GTP. These responses are markedly diminished by PTX (90% for L-alanine and 72% for L-arginine). PTX-catalyzed ADP-ribosylation in the presence of 32P-NAD indicated that the cilia contain a single PTX substrate of 40 kDa. Immunoblotting studies using an antiserum to the carboxyl terminal decapeptide of Gi1 and Gi2 showed that the 40 kDa PTX substrate cross-reacted with the anti-serum, indicating that it corresponds to Gi1 and/or Gi2.

Molecular cloning techniques are being used to identify transduction proteins involved in phosphoinositide hydrolysis in olfactory cilia. The expression and molecular nature of three target proteins within olfactory neurons are being studied: the 40 kDa PTX substrate, phospholipase C and protein kinase C. A cDNA library has been constructed from poly (A)<sup>+</sup> RNA isolated from olfactory rosettes. The average insert size of the library is 1.3±0.81 kbp. Oligonucleotide probes to three isoenzymes of phospholipase C have been obtained from NEN. These probes were labeled with digoxigenin-11-dUTP by tailing with terminal transferase. The labeled probes are now being used on Northern blots and to screen the cDNA library to identify the phospholipase C isoenzyme(s) expressed within olfactory neurons.

SIGNIFICANCE: Since only a single PTX substrate is expressed in the cilia from the catfish, this model system may provide a unique opportunity to identify the G-protein linking olfactory receptors to phospholipase C. Successful identification of the relevant G-protein would be the first investigation of this issue in olfaction. The successful construction of a cDNA library from olfactory epithelium will significantly facilitate studies of the expression and molecular nature of transduction protein mediating phosphoinositide hydrolysis in olfactory neurons.

WORK PLAN (next 12 months): We will continue our studies on the molecular nature of the 40 kDa PTX substrate using biochemical, immunohistochemical and molecular cloning techniques. An anti-serum that recognizes this polypeptide on Western blots will be used to inhibit stimulus-dependent IP3 formation in isolated cilia. The antiserum will also be used to immunoprecipitate the G-protein phospholipase C complex and to study the cellular localization of the 40 kDa PTX substrate by immunohistochemistry. If these studies confirm the involvement of this G-protein in mediating phosphoinositide hydrolysis, the protein will be isolated, partially sequenced and cloned. We will also continue the screening of Northern blots and cDNA libraries to identify other transduction proteins expressed in olfactory neurons.

PUBLICATIONS AND ABSTRACTS:

1. Abogadie, F.C. and Bruch, R.C. (1991) A pertussis toxin-sensitive G-protein may mediate phosphoinositide hydrolysis in olfactory cilia. Chem. Senses Abstract in press.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1583

R&T CODE: 441R010--01

PRINCIPAL INVESTIGATOR: John T. Caprio

INSTITUTION: Louisiana State University

Grant Title: Mechanisms of Vertebrate Olfactory Receptor Responses to Stimulus Mixtures

REPORTING PERIOD: 1 June 1990 - 31 May 1991 (12 months)

AWARD PERIOD: 15 March 1990 - 14 March 1993

OBJECTIVE: For the channel catfish, *Ictalurus punctatus*, determine: (A) how olfactory receptor neurons respond to mixtures of amino acid stimuli, and (B) the function of smell versus taste in behavioral responses to amino acid stimuli.

ACCOMPLISHMENTS (last 12 months): (A) Electrophysiology: Electrophysiological recordings of the electro-olfactogram (EOG) and neural activity from small populations of olfactory receptor neurons in response to complex mixtures (from 2 to 10 components) of amino acids were performed. The responses to the stimulus mixtures composed of a) neutral and acidic, b) neutral and basic, and c) acidic and basic amino acids were significantly enhanced compared to those mixtures consisting of an equal number of only neutral amino acids. These results demonstrate that receptor sites for acidic, basic and neutral amino acids, respectively, are highly independent from each other and suggest that a mechanism for synergism is the simultaneous activation of relatively independent receptor sites by the components in the mixture. Based on the dose/response relationship for individual amino acids, the enhancement observed for the neural activity to these mixtures (a-c) was equivalent to elevating the stimulus concentration of a single component approximately 50-fold.

(B) Behavior: Naive channel catfish responded to L-alanine and L-arginine ( $>1\mu\text{M}$ ) and L-proline ( $>1\text{mM}$ ) with the following patterns of feeding behavior: maxillary and mandibular barbel movements, orienting posture, search swimming, bottom and wall searching, turning and snapping. Catfish were subsequently conditioned during 40 trials to swim 90 seconds for a food reward following an application of the amino acid stimulus. The conditioned "search swim" consisted of 40-90 turns greater than  $90^\circ$  compared to 0-40 turns in response to non-conditioned amino acids. Conditioned channel catfish discriminated L-alanine, L-arginine and L-proline from each other and from all non-conditioned amino acids. Following surgical removal of the olfactory organs, animals previously conditioned to  $10^{-5}\text{M}$  L-proline did not respond behaviorally to L-proline at concentrations  $<10^{-4}\text{M}$ , whereas strong snapping behavior was triggered by  $10^{-3}\text{M}$  L-proline. L-arginine and L-alanine at  $10^{-5}\text{M}$  released short-



lasting feeding responses in the anosmic catfish; however, even after 45 - 150 re-conditioning trials the anosmic catfish did not show discrimination between the tested amino acids.

**SIGNIFICANCE:** (A) Electrophysiology: (1) Knowledge of the relative independence of receptor sites for the components in a stimulus mixture is essential for predicting olfactory receptor activity; (2) Olfactory receptor neural activity can be significantly enhanced by simply stimulating receptors with an appropriate amino acid mixture without elevating stimulus concentration.

(B) Behavior: 1) the conditioning to amino acid stimuli was a result of olfactory input; 2) the increased food search in catfish cannot be conditioned by taste alone; 3) the taste mediated responses to amino acids including snapping are most probably innate.

#### WORK PLAN (next 12 months):

1. A major objective of this period is to obtain information on the response properties of single olfactory neurons to stimulus mixtures in order to compare our results with integrated neural data obtained from small populations of receptor neurons and from the electro-olfactogram (EOG). We will continue recording from single mitral cells within the olfactory bulb simultaneously with EOG recordings according to our original proposal. We are also initiating a patch clamp study of isolated catfish olfactory receptor neurons to determine how individual receptor cells respond to both free amino acids and their simple mixtures. In collaboration with Richard Bruch, we will test the effects of pertussis toxin on olfactory receptor responses to amino acids that activate different olfactory receptor sites in both *in vivo* (EOG and neural recordings) and isolated cell (patch) preparations. Recent evidence by Bruch indicates that pertussis toxin inhibits IP3 formation in response to amino acid stimulation of isolated cilia from the olfactory organ of the channel catfish. We will also continue with the behavioral experiments to determine whether the enhanced olfactory activity observed electrophysiologically in response to particular stimulus mixtures is correlated with increased chemosensory behavioral patterns.

#### **PUBLICATIONS AND REPORTS:**

1. Kang, J. and Caprio, J. Submitted. Electro-Olfactogram and Multiunit Olfactory Receptor Responses to Complex Mixtures of Amino Acids in the Channel Catfish, *Ictalurus punctatus*. J. Gen. Physiol.
2. Valentincic, T., Wegert, S. and Caprio, J. Submitted. Taste mediates innate feeding behavior while olfaction facilitates conditioned discrimination of amino acids by catfish. Soc. Neurosci. abs.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1710

R&T CODE: 441r0008

PRINCIPAL INVESTIGATOR: Dr. Vincent E. Dionne

INSTITUTION: University of California San Diego

GRANT TITLE: Molecular Mechanisms of Olfactory Transduction

REPORTING PERIOD: 1 May 1990 - 30 April 1991 (12 months)

AWARD PERIOD: 1 April 1990 - 31 March 1993

OBJECTIVE: To identify and characterize molecular constituents of the olfactory transduction pathway.

ACCOMPLISHMENTS (last 12 months): We have attempted to reconstitute olfactory transduction pathways by expression of olfactory mRNA in *Xenopus laevis* oocytes. The studies used mRNA which we isolated from salmonid fish olfactory epithelia. Fish were chosen for the study for technical reasons: (1) their native odors have been well documented and include amino acids; (2) amino acids are water soluble; (3) amino acids have been shown to stimulate the inositol phospholipid second messenger system in fish olfactory tissue; (4) preliminary results had established the feasibility of the approach.

Olfactory tissue was collected from 200 farm-raised rainbow trout, *Salmo gairdneri*, and stored at -70C. Poly(A)<sup>+</sup>RNA was prepared by the batch-extraction method of Badley et al. (Biotechniques 1988, 6:59-61) and resuspended at 1µg/µl in H<sub>2</sub>O. An RNA gel showed staining from approx. 1kb to >10kb. However, in contrast to preliminary results, defolliculated oocytes, when injected with this RNA preparation and incubated for 3-6 days, were no more sensitive to test amino acids than uninjected control cells. The amino acids tested were the L-isomers of threonine, histidine, arginine, alanine, serine and valine. Small voltage-insensitive currents were reliably detected in both injected and uninjected oocytes; these may be due to an uptake mechanism. Additional poly(A)<sup>+</sup>RNA was prepared and size-fractionated on an agarose gel, but the fractions failed to express also. Co-expression studies using rat whole-brain RNA or a cloned *Xenopus* K<sup>+</sup> channel have shown that the trout RNA, prepared on several different occasions and by 2 different methods, suppresses expression of the other message. The mechanism of suppression is being examined.

Separate studies using odor-imprinted salmon were initiated in collaboration with G. Nevitt and A. Dittman (U. Washington, Seattle, WN). Hatchery-grown juvenile animals were imprinted with phenylethyl alcohol (PEOH) then farm-raised to adulthood. Olfactory tissue from these animals was used to prepare poly(A)<sup>+</sup>RNA by batch-extraction. The RNA was then injected into oocytes for expression. Initial results showed that PEOH suppressed a K<sup>+</sup> conductance in a dose-dependent manner at concentrations as low as 1µM, while control oocytes were insensitive to PEOH. However, only 10-20% of cells were responsive in the best injected batches, and often no cells responded. This percentage of responsive cells is too low for reliable study; we are examining possible solutions. We believe that odor transduction pathways are comprised of a large number of constituents (receptors, G-proteins, enzymes and ion channels), all of which must be present for functional expression. This requirement reduces the likelihood of expressing the pathway in tact.

A separate series of studies was begun using rat olfactory tissue. Complimentary DNA derived from rat olfactory epithelium was amplified by PCR with highly degenerate pairs of oligonucleotides from the 3rd and 7th

transmembrane regions of the general class of 7-Transmembrane Domain (TMD) receptors. This allowed us to identify a multigene family of putative olfactory 7-TMD receptors. *In situ* analysis with one of them (OR3) shows a pattern of expression in the olfactory epithelium with no expression in other tissues.

**SIGNIFICANCE:** We believe that odor transduction involves 1) a multitude of olfactory 7-TMD receptors coupled to either CAMP or IP3, and 2) groups of functionally related olfactory neurons distributed in clusters in the olfactory epithelium. Our evidence supports this perception, but it is not conclusive.

**WORK PLAN (next 12 months):** We will prepare cDNA libraries from trout and salmon olfactory tissue and probe them for sequences homologous to those isolated from the rat. We will pursue studies to test whether the family of 7-TMD receptors are functional olfactory receptor proteins or whether they have other physiological roles. We will examine additional members of the family of 7-TMD receptors that we have already isolated and test whether their distributions are restricted to olfactory receptor neurons.

**PUBLICATIONS AND ABSTRACTS (last 12 months):**

P. Nef, S. Heinemann and V.E. Dionne. Spatial distribution of OR3, a putative seven-transmembrane domain olfactory receptor, reveals an olfactory map. *ACheMS* (1991) 13:170 (Abstract)

P. Nef, I. Hermans-Borgmeyer, G.P. Gasic, S. Heinemann and V.E. Dionne. Discrimination of odors by 7-TM domain receptors and by a group of olfactory neurons. *Society of Neuroscience Abstracts* (1991) (submitted)

## ANNUAL PROGRESS REPORT

GRANT#: N00014-90-J-1432

R&T CODE: 441r001

PRICIPAL INVESTIGATOR: Dr. Stuart Firestein

INSTITUTION: Yale University Medical school

GRANT TITLE: Basic Mechanisms of Transduction in  
Olfactory Sensory Neurons

REPORTING PERIOD: 1 June 1990 - 31 May 1991 (12 months)

AWARD PERIOD: 15 January 1990 - 14 January 1994

OBJECTIVE: To investigate the molecular steps underlying odor detection, discrimination, and the generation of an electrical sensory signal in olfactory receptor neurons.

ACCOMPLISHMENTS: A final, detailed study on the second messenger system underlying transduction has been completed. These experiments allowed us to propose a physiological model for transduction and to suggest rate limiting and amplification steps in both the onset and decay phases of the odor response.

In collaboration with Dr. Frank Zufall we have completed a comprehensive study of the cAMP gated channels in olfactory receptors. The results of this study provide direct and conclusive evidence that the odor sensitive current is identical to the current through cAMP gated channels. Further we have been able to fully characterize the behavior of single channels, both in response to odors and to direct application of cyclic nucleotides. This analysis has uncovered a novel mechanism of Ca++ dependent desensitization that could not have been seen at the level of macroscopic current recording.

SIGNIFICANCE: These data enable us to understand the events leading to signal generation which are common to most or all odor ligands. This is the beginning of a more quantitative approach to describing signal transduction in this system.

WORK PLAN: (next 12 months): We will continue to investigate the behavior of the cyclic nucleotide gated channel, which we are now able able to record on a regular basis. This channel occupies a critical position in the transduction process since it is the final step in the biochemical enzyme cascade and the first step in the generation of the electrical signal. Detailed kinetic

investigations will be undertaken with rapid agonist application techniques, such as caged nucleotides.

Concurrent with these experiments we will begin a series of experiments devoted to determining precise dose-response relations for a wide range of cells utilizing odor mixtures and individual components. It is expected that these studies will produce information regarding the physiological basis of sensitivity and selectivity in these cells.

Finally a detailed computer model of the second messenger system is being prepared based on the experimental data developed this past year. This will utilize a combination of standard engineering software (SABRE) and newly developed simulation programs (NEURON) running on a DEC 5000/200 workstation. We believe this modelling effort will enable us to provide some important insights into the general questions involving signal transduction in G-protein coupled systems. The olfactory receptor is rapidly becoming an important model system for addressing these issues.

#### PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Firestein, S., Darrow, B. & Shepherd, G.M. (1991)  
Activation of the sensory current in salamander olfactory receptor neurons depends on a G-protein mediated cAMP second messenger system. *Neuron* 6: 825-835.
2. Zufall, F., Firestein, S. & Shepherd, G.M. (1991)  
Single channel recordings of cyclic nucleotide gated, odour sensitive channel in isolated salamander olfactory receptor neurones. *Physiological Society Meetings* (London) March 1991.
3. Firestein, S., Zufall, F. & Shepherd, G.M. (1991)  
Odor induced single channel; activity in membrane patches from salamander olfactory receptor neurons. *ACHems* 13, Sarasota FL.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1445

R&T CODE: 441r007

PRINCIPAL INVESTIGATOR: Stephen P. Fracek Jr.

INSTITUTION: University of North Texas

GRANT TITLE: A Neural Network Model of Olfaction

REPORTING PERIOD: 1 June 1990 - 31 May 1991 (12 months)

Award Period: 1 January 1990 - 31 December 1992

OBJECTIVE: To develop a biological neural network consisting of dissociated, cultured mouse olfactory bulb cells co-cultured with olfactory mucosal explants that may be capable of information processing and odorant discrimination; to characterize this system in terms of its electrophysiology, pharmacology, histology, immunohistology, and response to odorants; to test theoretical network models against this biological model.

ACCOMPLISHMENTS (last 12 months): We have shown that these cultures contain cells that are ultrastructurally similar to mitral/tufted cells, granule cells, short axon cells, and astrocytes. The cultures consist of individual cells and aggregates. Large fascicles connect the aggregates; individual cells have extensive neurites. There are many synapses, especially in the aggregates; some of the synapses are reciprocal. Three types of synaptic vesicles are present: electron translucent, dense core, and coated. Various populations of these cells stain with antibodies to MAP2, Tau, NSE, NF200, GABA, GAD, and taurine. Antibodies to aspartate do not stain these cultured cells. Antibodies to dopamine and glutamate yield ambiguous results.

Current computer algorithms are adequate for burst identification but are incapable of action potential identification. Amplitude decay during a burst and fasciculation causes part of this problem. There are few commercially available programs that suit our needs or have the capacity to handle our large data files. Consequently, we have had to design and implement specialized data analysis programs in the computer language C and the various UNIX shell languages. Some of these programs include: cumulative real time histograms, which are useful for determining noise and signal voltage amplitudes; programs for compressing data as it comes off the A/D boards; and burst identification and characterization modules.

The cultures form spontaneously active networks; the most common form of electrophysiological activity is bursting. The electrophysiological activity is spatially and temporally complex. This activity can be reversibly manipulated with pharmacological agents (e.g. GABA, bicuculline, picrotoxin); the response to these agents is similar to that seen in the

intact animal. These agents cause the network activity to change states. The differential effects of pharmacological agents among the channels may indicate the presence of granule cells and periglomerular cells. These networks show some characteristics (e.g. sensitivity to initial conditions) of chaotic dynamics.

SIGNIFICANCE: Cultured olfactory bulb cells form neural networks. These cultures have some of the characteristics of the intact bulb based on ultrastructural, immunocytochemical, pharmacological, and electrophysiological evidence. Thus, the cultured networks may be capable of information processing and odorant detection.

WORK PLAN (next 12 months): We will complete our morphological and immunohistochemical comparison of cultured olfactory bulb cells with intact bulbs from embryos, neonates, and adults using antibodies to the following: MAP2, Tau, GFAP, NSE, NF200, GABA, GAD, aspartate, dopamine, glutamate, and taurine.

We are implementing the algorithms outline by Shaw (The Dripping Faucet as a Model Chaotic System, Aerial Press, Santa Cruz, CA, 1986) for data analysis using information metrics. We are developing techniques that will allow us to identify individual action potentials.

We are analyzing our data to determine if information is conserved in time and for how long. We will determine if cell populations can be identified based on the information content of their electrophysiological activity.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Weil, J. and Fracek, Jr., S.P. (1990) GABAergic circuits of dissociated mouse olfactory bulb cell cultures. Soc. Neurosci. Abstr. 16: 403.
2. Guo, L., Thomas, T., Schafer, R., and Fracek, Jr., S. P. (1990) Immunocytochemistry of dissociated mouse olfactory bulb cell cultures. Soc. Neurosci. Abstr. 16: 403.
3. Fracek, Jr., Stephen, Weil, J., Schafer, R., and Gross, G.W. Cultured Olfactory Neuronal Networks: Spontaneous Multisite Activity. Submitted to Brain Research Bulletin and currently undergoing revisions.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1744

R&T CODE: 4414906

PRINCIPAL INVESTIGATOR: William F. Gilly

INSTITUTION: Stanford University, Hopkins Marine Station

GRANT TITLE: Chemoreception In Squid And Its Role In Controlling Specific Motor Systems Responsible For Jet-Propelled Swimming And Escape Responses

REPORTING PERIOD: 1 June 1990 - 31 May 1991 (12 months)

AWARD PERIOD: 1 May 1989 - 30 April 1992

### OBJECTIVE:

1. Define chemoreceptive abilities of squid to stimuli which produce (or inhibit) specific motor outputs. Identify other substances which may be biologically important for other functions.
2. Characterize the relevant chemosensory cells using electron microscopic and patch voltage clamp methods. Voltage-, transmitter- and second messenger-regulated channels will be studied in order to form a complete picture of each type of receptor cell.
3. Elucidate mechanisms of detection and transduction of specific stimuli (identified by Objective 1) in receptor cells using biochemical and voltage clamp methods to study modulation of the channels studied in Objective 2.
4. Explore integration of chemosensory information by two defined motor pathways that control jet propulsion. Investigate interactions between olfaction and other sensory modalities in controlling these motor outputs.
5. Identify the molecules in which biological activity resides in natural products to be studied which produce strong behavioral responses.

ACCOMPLISHMENTS (LAST 12 MONTHS): We have made considerable progress on many of the objectives listed above. Accomplishments in each area will be discussed separately.

1. We performed a series of behavioral experiments aimed at identifying biologically relevant substances that produce specific motor outputs when applied to squid olfactory organ. We tested extracts from various squid organs, ie. nidamental gland, accessory nidamental gland, squid brain, mantle, testes, ovary, intestines, and ink sac. We found that of all these extracts, only squid ink causes escape jetting in behavioral experiments. L-DOPA, a precursor of the melanin pigment in squid ink, also elicits escape jetting. In addition, behavioral experiments using quaternary ammonium compounds were completed (Gilly & Lucero, 1991).

2. The two most common receptor cell types (pyriform and flori-form) have been characterized by SEM, TEM, and light microscopy. We have submitted a manuscript on the responses of isolated receptor cells to chemical stimulation by quaternary ammonium compounds and squid ink (Lucero, Horrigan, & Gilly, 1991).

3. We have been successful at using the 'perforated patch' technique to record from the isolated receptor cells in current clamp. This technique allows us to study the cells with second messenger systems intact. We found that application of low concentrations of squid ink, L-DOPA, or dopamine hyperpolarize the membrane potential and decrease excitability of the receptor cell. This hyperpolarization is associated



with an increase in membrane conductance due to the opening of K or Cl channels. We have also completed the studies outlined last year on the effects of quaternary ammonium ions on Na and K currents in these cells. We found that similar to the behavioral experiments, TEA and TBA have the strongest effects and block 20% of the outward K current (without affecting Na current) when "puffed" onto the cells. TMA had no effect on either Na or K currents (Lucero, Horrigan, & Gilly, 1991).

4. We have developed a system for making electrical recordings from the area of the stellate ganglion of living hatchling squid. We are able to resolve activation of both the giant and small motor pathways when using electrical, visual, or tactile stimulation. This system will be very useful for investigating interactions between olfaction and other sensory modalities in controlling these motor outputs.

5. The main natural product that was studied this past year was squid ink. We have identified that both L-DOPA and dopamine mimic the effects of squid ink when applied to the isolated receptor cells.

**SIGNIFICANCE:** A major difficulty in characterizing chemosensory capabilities in a new organism is to identify a biologically relevant stimulus that elicits a characteristic behavioral response. The most significant aspect of our work this past year was to show that squid ink causes escape jetting responses and affects the excitability of isolated receptor cells. With a biologically relevant stimulus in hand, we will be able to make rapid progress on all aspects of the project.

**WORK PLAN (next 12 months):** The objectives for next year will again be directed toward both isolated receptor cells and whole living squid.

**1. Experiments on isolated receptor cells.**

A primary goal will be to determine the transduction mechanism involved in the response to squid ink stimulation. First, we will determine the ionic basis of the conductance increase. Second, the possibility that the squid ink response is mediated via a dopaminergic receptor will be explored by using dopamine receptor specific agonists and antagonists on the isolated receptor cells. If a dopaminergic receptor is involved, we will try to clone the receptor using PCR. We will also perform labeling studies to visualize the location of the receptor in the olfactory neurons. In addition, we will complete a manuscript describing the biophysical properties of the ion channels present in the two main olfactory cell types.

**2. Experiments on living squid.**

We plan to make use of the *in vivo* prep for recording both giant and small motor activity. We will compare the data that we are currently gathering on electrical, visual, and tactile stimulation with responses to chemical stimulation. Differences between motor output in response to activation of the various sensory systems (ie. latencies, threshold, order of activating giant vs small motor fibers) will give clues to the level of processing occurring within each system.

**PUBLICATIONS AND ABSTRACTS (last 12 months):**

1. Lucero, M.T., F.T. Horrigan, J.L. Levitt, & W.F. Gilly. (1991) Chemo-receptive capabilities of the squid olfactory organ. Abstract presented 26 Feb. 1991, *Biophysical Journal*. 59:184a.

2. Lucero, M.T., F.T. Horrigan, and W.F. Gilly. 1991. Electrical Responses to chemical stimulation of isolated squid olfactory receptor cells. Submitted to *J.Exp. Biol.*

3. Gilly, W.F. and M.T. Lucero. 1991. Behavioral responses to chemical stimulation of the olfactory organ of the squid *Loligo opalescens*. Submitted to *J. Exp. Biol.*

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1560

R&T CODE: 441r003

PRINCIPAL INVESTIGATOR: Dr. John S. Kauer

INSTITUTION: New England Medical Center Hospitals, Inc.

GRANT TITLE: Development of a Mathematical Description of the Olfactory Bulb, Validated with Intracellular and Voltage-Sensitive Dye Recordings.

REPORTING PERIOD: 1 July 1990 - 12 June 1991

OBJECTIVE: The major objective of this work is to develop a detailed mathematical description of the function of the olfactory bulb of the tiger salamander. This work will especially focus on incorporating into the model physiological responses of single cells observed by intracellular recording and of the entire network by video-rate imaging of voltage-sensitive dye fluorescence.

ACCOMPLISHMENTS (last 12 months): We have developed a mathematical model of the salamander olfactory bulb which incorporates all the known anatomical and physiological characteristics of this structure that are available. The equations we have written are designed to calculate the membrane potential at the cell body of each of the major neuron types in the olfactory epithelium and bulb. Outputs from the model are generated when these equations are calculated iteratively over time. The format of the output consists of continuous graphs of membrane potential for selected cells (similar to intracellular recordings). As one can see from the output demonstrated in the reprint, the model generates responses that are strikingly similar to the complicated membrane potential and spiking patterns seen in the real salamander olfactory bulb.

SIGNIFICANCE: The use of a mathematical model to represent the anatomical and physiological information available on the peripheral olfactory pathway allows us to: 1) formally organize these data in a way that permits assessment of whether sufficient information is available to represent the function of the system in mathematical terms; 2) test various perturbations of the model system and to examine the same perturbations in the animal; 3) examine whether the model is able to perform odor recognition with the same degree of sensitivity and discrimination as the real system, and, if so, to serve as a guide for building an artificial chemosensor.

WORK PLAN (next 12 months): We have reached the first plateau in the design and building of the model in that we have a complex functioning system which replicates, in

significant detail, the responses of the salamander olfactory epithelium and bulb. We now plan to continue to develop the details of the model by adding components of the neuronal network not yet included and to examine the degree to which each of the components contribute to the final output.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. White, J., S.R. Neff, K.A. Hamilton, J.S. Kauer. (1991) Emergent properties of odor information coding in a representational model of the salamander olfactory bulb. J. Neurosci. (submitted)

2. White, J., Neff, S.N. Cinelli, A.R., Kauer, J.S. (1990) Modelling the salamander olfactory bulb: single cell and network interactions. Neurosci. Abst., abstract.

3. White, J., S.N. Neff, A. Cinelli, J.S. Kauer. (1991) A large-scale computer model of the salamander olfactory bulb: responses to simulated electrical and odor stimulation. ASChS Absts.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1960

R&T CODE: 4414910

PRINCIPAL INVESTIGATOR: Michael Leon

INSTITUTION: University of California, Irvine

GRANT TITLE: Responses of Olfactory Projection Regions

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 July 1989 - 30 September 1991

OBJECTIVE: To investigate the role of afferent and efferent connections of the olfactory bulb during and following early olfactory learning.

ACCOMPLISHMENTS: We have completed an analysis of the responses of olfactory bulb projection areas to an odor that young rats have learned to prefer, with particular emphasis on the anterior olfactory nucleus and piriform cortex, but including a wide range of brain regions, using computer-assisted image analysis of  $^{14}\text{C}$  labelled 2-deoxyglucose (2-DG) autoradiographs. In all cases there were no discriminable differences in 2-DG uptake in any of the brain regions studied, in contrast to the clear differential uptake in the bulb to the preferred odor.

Centrifugal inputs to the bulb appeared to be involved in such learning. We had previously shown that blocking beta noradrenergic receptors blocks the neural and behavioral consequences of early learning. Using in vivo microdialysis in awake 3-day old pups, and analyzing the material with high pressure liquid chromatography, we demonstrated that there is an increase in the amount of norepinephrine that evoked by the reinforcing tactile stimulation that is paired with an odor to produce a preference. Since there is repeated pairing of the odor with an evoked increase in noradrenaline over the course of training, we hypothesized that eventually, there would be a conditioned increase in noradrenaline in response to the odor alone. Indeed when odor was paired with tactile stimulation, there was a conditioned increase in the neurotransmitter on postnatal day 7 in response to the odor alone. The conditioned increase in neurotransmitter could be involved in the maintenance and/or expression of the response to learned odors.

We also have completed our study of dopamine changes in the bulb during learning. While we find no conditioning increase in dopamine, we do find that both odor and tactile

stimulation on postnatal day 3 increase dopamine significantly. The combined stimulation, however, results in a learned odor preference, as well as large and long-lasting increase in dopamine in the olfactory bulb.

SIGNIFICANCE: The 2-DG pattern suggests that we will need to use a different, perhaps more sensitive technique to reveal activity differences in the olfactory bulb projection areas. The increase in noradrenaline in the bulb during learning and in response to the learned odor further reinforce the importance of this centrifugal element in the development and expression of the neural and behavioral response to early learning. The potentiated dopaminergic response to the combined odor and reinforcing tactile stimulation may be an example of a Hebbian system that arises during early olfactory learning.

WORK PLAN: Within the next year, we hope to extend our analysis of the projection regions of the olfactory bulb, using in situ hybridization to c-fos, an immediate early gene that we have found to be more sensitive to neural stimulation than the 2-DG method. We will also determine whether there is a potentiated noradrenergic response to concurrent odor and tactile stimulation. Finally, we will determine whether the blocking effects of increased sensory stimulation are mediated by changes in noradrenaline.

PUBLICATIONS AND ABSTRACTS:

1. Coppersmith, R., Weihmuller, F., Kirstein, C.L., Marshall, J. F., and Leon, M. Extracellular dopamine increases in the neonatal olfactory bulb during odor preference training. *Developmental Brain Research*, submitted.
2. Leon, M., Wilson, D.A., and Guthrie, K.M. Plasticity in the developing olfactory system. In: *Olfaction as a Model System for Computational Neuroscience*. H. Eichenbaum and J. Davis, (Eds.), M.I.T. Press, in press.
3. Kirstein, C.L., Weihmuller, F.B., Marshall, J.F., and Leon, M. Conditioned norepinephrine response in the olfactory bulb in 7-day old rat pups. *Society for Neuroscience Abstracts* (in press).

## ANNUAL PROGRESS REPORT

GRANT N00014-89-J-3071

R&T CODE: 4414913

PRINCIPAL INVESTIGATOR: Thomas Hellman Morton, Ph.D.

INSTITUTION: University of California, Riverside

GRANT TITLE: Chemical Modification of the Olfactory Receptor  
Epithelium of Vertebrate Species

REPORTING PERIOD: 1 July 1990 to 30 June 1991

AWARD PERIOD: 1 July 1989 to 30 June 1992

OBJECTIVE: To develop methodologies for attachment of cell-surface binding sites to solid surfaces using hydrophilic tethers that incorporate reversible linkers.

ACCOMPLISHMENTS (last 12 months): Hydrophilic tether components have been synthesized and attached to solid surfaces. A microelectrode with <0.2s response time has been fabricated using this kind of tether to connect biotinylated horseradish peroxidase to a carbon fiber surface via an avidin-biotin-avidin sandwich. A hydrophilic tether containing a cleavable unit (a fluoridolizable linker) has been used to purify a covalently modified protein.

WORK PLAN (next 12 months): The objective of Year 3 is to prepare various acetoacetic esters (including one attached to the above-mentioned biotinylated tether component) and explore the labelling of Schiff base-forming sites via covalent modification using the acetoacetic ester plus a borohydride reagent. A high performance liquid chromatograph (HPLC) has been purchased to facilitate fresh purification of small quantities of these acetoacetic esters immediately prior to use and to separate and identify novel amino acids from modified proteins.

### PUBLICATIONS AND REPORTS (last 12 months):

1. Pantano, P., Morton, T.H. and Kuhr, W.G. (1991) Enzyme-Modified Carbon-Fiber Microelectrodes with Millisecond Response Times. J. Am. Chem. Soc., 113: 1832-1833.
2. Grishow-Phelan, L.E., Kelcher, M.G., and Morton, T.H. (1990) Irreversible Radiolabeling of Schiff Base-Forming Membrane Proteins in the Olfactory Epithelium of Tiger Salamanders. Paper was presented at the 20th Annual Meeting of the Society for Neuroscience, St. Louis, 31 October 1990.

# ANNUAL PROGRESS REPORT

**GRANT#:** N00014-90-J-1515 R&T Ccde 441r005

**PRINCIPAL INVESTIGATORS:** W.C. Neely, S.D. Worley, A.J. Illies

**INSTITUTION:** Auburn University

**GRANT TITLE:** Determination of the Extent and Mechanism of Binding of Different Classes of Odorant Molecules to Relevant Biological Substrates

**REPORTING PERIOD:** 1 June 1990 - 31 May 1991

**AWARD PERIOD:** 1 March 1990 - 28 February 1994

**OBJECTIVE:** To obtain an understanding of the biophysics of binding of odorant molecules to substrates relevant to the olfactory systems of invertebrates and to use this information to develop biological substrates for concentrating odorants so as to enhance odorant detection for trained animals and artificial devices.

**ACCOMPLISHMENTS (last 12 months):** Langmuir-Blodgett (LB) films have been used as models of biological substrates for odorant recognition studies. This work performed in collaboration with the Department of Physiology, has employed (+)- and (-)- carvone as odorants and 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC) as the model substrate and has demonstrated significantly greater interaction with the more intense odorant (-)-carvone than with the weaker odorant (+)-carvone. LB films have also been used to orient the peptide valinomycin which selectively binds potassium ions. The film becomes electrically polarized upon binding  $K^+$  and will be the basis of a sensor capable of analytically determining  $K^+$  in the presence of  $Na^+$ , a measurement of considerable biomedical importance. A combination of this sensor and a suitable amplifier on a single chip is being fabricated. This integrated transducer/amplifier approach is a model for future odorant sensors. Also, pressure/volume expansion measurements have continued for a series of model odorants and biological substrates. Thus far, the strongest interactions observed have been between benzaldehyde or methyl benzoate as odorants and lysine as a substrate. Mass spectrometry was employed to show that these interactions were reversible at ambient temperature. Self consistent fields molecular orbital calculations of the AM1 variety showed that binding between the odorant cocaine and the biological substrate glycine could occur through nucleophilic attack on the carbonyl groups of cocaine by the nitrogen lone pair on the amino acid. Also, the semiempirical AMYR method has been employed to predict binding energies and molecular orientations in the bound state for the model odorant cyclohexanone with the model substrate stearic acid. The computed interaction energy was -14.5 Kcal/mol in excellent agreement with reported experimental data (-12.6 and -11.9 Kcal/mol) given that the calculations refer to the gas-phase, while the experimental data were for film prepared on water.

SIGNIFICANCE: If the mechanisms in distinguishing various odorants by invertebrates can be determined utilizing biophysical chemistry methods such as those used in the work noted above, then a detection device can probably be designed based upon the concentration of the odorants on biological substrates, which will be of use to the law enforcement branch of the U.S. Navy.

WORK PLANNED (next 12 months): Work will be continued concerning binding of odorant molecules onto LB films as biological substrates or model compounds. Measurement of binding interactions will include both thermodynamic determinations (free energy, entropy, and enthalpy) and spectroscopic observations (fluorescence emission and infrared absorption). Further pressure/volume expansion measurements will be made to determine the optimum odorant/biological substrate interaction using model odorants and amino acids. The optimum interacting pairs will be examined by X-ray photoelectron spectroscopy to determine the natures of the binding sites. Computational methods (AM1 and AMYR) will be utilized to estimate relative interaction energies for various binding geometries so as to correlate with the experimental data in determination of the molecular mode of binding of the selected odorants and substrates. The results obtained in these studies will be used in developmental work for odor-discriminating sensors.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. C.S. Wu, W.C. Neely, and S.D. Worley, "A Theoretical Study of the Molecular Interaction of Cocaine with the Biological Substrate Glycine", J. Comp. Chem., in press.
2. C.S. Wu, W.C. Neely, and S.D. Worley, "Theoretical Study of the Interaction of Cocaine with Biological Substrates", Southeast/Southwest Regional American Chemical Society Meeting, New Orleans, LA, Dec. 1990.
3. V. Vodyanoy, S. Pathirana, L.J. Myers, and W.C. Neely, "Molecular Recognition of Optical Isomers (+) and (-) Carvone by Phospholipid Monolayers", 35th Annual Meeting of the Biophysical Society, San Francisco, CA, Feb. 1991.



### Annual Progress Report

GRANT #: N00014-90-J-1519

R & T CODE: 441r006

PRINCIPLE INVESTIGATOR: Dennis E. Rhoads, Ph.D.

INSTITUTION: The University of Rhode Island

GRANT TITLE: The Role of  $\text{Ca}^{2+}$  in Olfactory Transduction

REPORTING PERIOD: 1 June 1990 - 31 May 1991 (12 months)

AWARD PERIOD: 1 Mar 1990 - 28 Feb 1993

OBJECTIVE: To identify and to characterize the biochemical mechanisms responsible for: (a) odorant reception and (b) control of  $\text{Ca}^{2+}$  activity by receptor cells in the olfactory rosettes of Atlantic salmon.

ACCOMPLISHMENTS (last 12 months): We have completed studies on the characterization of the ligand binding properties of a receptor for the odorous amino acid, L-alanine. An affinity ( $K_D = 3 \mu\text{M}$ ) relevant to electrophysiological and behavioral responses to L-alanine, specificity for short chain neutral amino acids with clear discrimination against basic and acidic amino acids, and the pattern of inhibition by metal ions and reduced pH, all attest to the physiological relevance of this binding site in olfactory discrimination. Interestingly, less than 15% of the L-alanine binding sites present in the rosette are recovered in cilia preparations. Thus, in Atlantic salmon there is a substantial role of nonciliary membrane in olfactory reception and we propose that microvillous rather than ciliated receptor cells may play the major role in L-alanine reception. With plasma membrane rich fractions from the olfactory rosettes (OR), L-alanine causes a specific increase in generation of inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) that is rapid and transient. These, initial studies suggest that L-alanine reception involves the  $\text{Ca}^{2+}$ -mobilizing transduction system associated with enzymatic hydrolysis of phosphatidylinositol 4,5-bisphosphate ( $\text{PIP}_2$ ) to produce  $\text{IP}_3$  and, very likely, other active metabolites.

Levels of divalent cation dependent ATP phosphohydrolase (ATPase) activities were measured in cilia and other plasma membrane rich fractions from the salmon OR. In cilia preparations, the amount of  $\text{Ca}^{2+}$ -ATPase activity ( $V_{\text{max}} = 1.1 \mu\text{mole Pi/min/mg protein}$ ) and the apparent affinity of the enzyme for  $\text{Ca}^{2+}$  ( $K_m = 10\text{nM}$ ) are among the highest reported in any membrane system.  $\text{Ca}^{2+}$ -ATPase activity in other plasma membrane rich fractions had an identical  $K_m$  but lower  $V_{\text{max}}$ . Endogenous calmodulin appeared to associate tightly with olfactory membranes and to increase  $\text{Ca}^{2+}$ -ATPase activity because calmodulin antagonists effectively inhibited the enzyme even after

multiple washes of membrane preparations with buffers containing EGTA. In EGTA treated preparations,  $\text{Ca}^{2+}$ -ATPase was stimulated by addition of bovine calmodulin but only at  $\text{Ca}^{2+}$  concentrations less than 10nM.

SIGNIFICANCE: The identification of a putative  $\text{Ca}^{2+}$ -mobilizing receptor system will facilitate studies aimed at direct investigation of  $\text{Ca}^{2+}$  flux and at the effects of elevated intracellular  $\text{Ca}^{2+}$  in olfactory receptor cells. The very high affinity and capacity of the olfactory  $\text{Ca}^{2+}$ -ATPase activity establish this enzyme, which provides active transport of  $\text{Ca}^{2+}$ , as the prime candidate for control of intracellular (including intraciliary) free  $\text{Ca}^{2+}$  in this system. Activity and regulation of this enzyme may be essential features of  $\text{Ca}^{2+}$  homeostasis in olfactory sensory cells.

WORK PLAN (next 12 months): During the next year we will continue characterization of odorant stimulated  $\text{PIP}_2$  metabolism and focus on  $\text{Ca}^{2+}$  influx,  $\text{Ca}^{2+}$ /calmodulin dependent protein kinase, and protein kinase C. In addition, we will expand studies of the relative contributions of cilia and nonciliary plasma membrane in reception to include basic and acidic amino acids and non-amino acid odorants. Within this context, we also intend to examine the extent to which any of these other odorants activate adenylate cyclase so that potential interactions between  $\text{Ca}^{2+}$  and cAMP can begin to be evaluated.

PUBLICATIONS AND ABSTRACTS (last 12 months):

Lo, Y.H., Bradley, T.M., and Rhoads, D.E. (1991) L-alanine binding sites and  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase in cilia and other membrane fractions from olfactory rosettes of Atlantic salmon. Comp. Biochem. Physiol. 98B:121-126.

## ANNUAL PROGRESS REPORT

Grant #: N00014-90-J-1056

R&T CODE

4411107

PRINCIPAL INVESTIGATOR: Dr. S. A. Simon

INSTITUTION: Duke University

GRANT TITLE: Functional Reconstitution of the Olfactory Membrane

REPORTING PERIOD: 1 October 1989 - 31 December 1990

AWARD PERIOD: 1 October 1989 - 31 December 1990

OBJECTIVE: To understand at a molecular level the mechanisms underlying olfactory transduction.

ACCOMPLISHMENTS: We have made substantial progress toward the initial goals of our proposal, i.e., the study of odorant activated ion channels. We have incorporated chemosensory cilia from olfactory receptor cells of the bullfrog, *Rana catesbeiana*, in planar lipid bilayers and characterized the concentration dependence and voltage dependence of currents induced by the bellpepper odorant, 3-isobutyl, 2-methoxypyrazine (IBMP). We recorded current-voltage curves between -100 mV and +100 mV at 20 mV intervals with 5 s at each voltage and a 1.5 s rest at 0 mV between each voltage step. Successive current-voltage curves were recorded at 1 to 5 min intervals. This procedure was repeated at different concentrations of odorant. Nanomolar concentrations of IBMP generated a dose-dependent increase in the steady state transmembrane current. The dose-response curve saturates near 500 nM and activation of the current is half-maximal at 75 nM IBMP. The nanomolar potency with which IBMP stimulates the transmembrane current under our conditions together with the saturating behavior of the dose-response curve indicates that the action of IBMP is specific and mediated via a limited number of high affinity sites.

Current-voltage curves are linear at all odorant concentrations, but the current fluctuations show marked asymmetry in their voltage dependence with larger amplitudes of fluctuations at increasing positive voltages. Thus, both the concentration of odorant and the membrane potential are important in controlling the activity of odorant-gated conductance pathways.

To assess the nature of the macroscopic currents we decided to characterize the single channels that underlie these odorant-induced currents. Although single channels could be recorded in conventional planar lipid bilayers, it was difficult to change solutions in this system and bilayers were frequently unstable. Therefore, we adopted a method of forming planar bilayers on the tips of patch pipettes. These bilayers were more stable, showed greater reproducibility and, hence, were better suited for single channel recording. Using this method, we recorded single channels at voltages ranging from -160 mV to +160 mV in symmetrical solutions containing 0.2 M NaCl. In the absence of odorants, single channels were observed with a conductance of 100 pS. The current-voltage relationship was linear with a reversal potential at 0 mV. Future studies will focus on characterizing channels that appear upon addition of odorants and the probability of opening and amplitude of these channels in the presence of different concentrations of odorants will be compared to the dose-dependence of the odorant-induced macroscopic currents described above.

SIGNIFICANCE: We have generated information on the dose-dependence and voltage-dependence of macroscopic odorant-induced currents.

WORK PLAN: (next 12 months even though Grant is terminated)  
The experiments which will be conducted during the renewal period of this grant will be a logical continuation of the studies performed during the preceding period, namely a detailed characterization of odorant-activated ion channels at the single channel level. As mentioned above, we will record single channels in planar bilayers formed over the tips of patch pipettes and study the probability of opening and amplitude in the presence of different concentrations of IBMP over a range of voltages. We will then be able to evaluate the single channel basis of the odorant-induced macroscopic currents documented during the preceding grant period.

In addition, we will start to characterize cyclic nucleotide-gated ion channels in olfactory receptor cells. One prominent transduction mechanism by which odorants signal excitation of olfactory receptor neurons consists of G-protein mediated activation of adenylate cyclase and subsequent opening of cyclic nucleotide-regulated channels, that are gated directly by cyclic AMP and that are homologous to cyclic GMP-gated channels of photoreceptor cells. We will study ion permeation and gating properties of cyclic nucleotide-activated channels in planar lipid bilayers and in olfactory receptor neurons and compare them with cyclic GMP-activated channels in photoreceptor cells. The aim of these experiments will be to understand the mechanisms of ion permeation through these channels, in particular the

interactions between monovalent and divalent cations. These experiments will lead to a better understanding of cyclic nucleotide-activated channels in olfactory receptor neurons.

#### PUBLICATIONS AND ABSTRACTS

Labarca, P., Simon, S. A. and Anholt, R. R. H. (1988)  
Activation by odorants of a multistate cation channel from  
olfactory cilia. Proc. Natl. Acad. Sci. U.S.A. **85**: 944-947.

Anholt, R. R. H., Petro, A. E. and Rivers, A. M. (1990)  
Identification of a group of novel membrane proteins unique  
to chemosensory cilia of olfactory receptor cells.  
Biochemistry, **29**: 3366-3373.

Anholt, R. R. H. (1989) Molecular physiology of olfaction.  
Am. J. Physiol. **257** (Cell Physiol **26**): C1043-C1054.

Anholt, R. R. H., Farmer, R. W. and Karavanich, C. A. (1989)  
Excitation by odorants of olfactory receptor cells:  
Molecular interactions at the ciliary membrane. In: Chemical  
Senses: Molecular Aspects of Taste and Odor Reception  
(Editors, J. G. Brand, J. H. Teeter, M. R. Kare and R. H.  
Cagan) Chapter 17, pp. 347-361, Marcel Dekker, Inc., New  
York, NY.

Anholt, R. R. H. (1991) Molecular aspects of olfaction. In:  
Olfaction and the Central Nervous System (Editors, M. Serby  
Verlag and K. Chobor), Springer Verlag, in press.

Labarca, P., Simon, S. A. and Anholt, R. R. H. (1986)  
Activation by odorants of a voltage-dependent, cation-  
selective channel in ciliary membranes from the olfactory  
epithelium of the bullfrog. Soc Neurosci. Abst. **12**: 1178.

Labarca, P., Simon, S. A. and Anholt, R. R. H. (1987) Ion  
channels of olfactory cilia. Ninth Annual Meeting of the  
Association for Chemoreception Sciences, Abst. 60.

**SENSORY BIOPHYSICS IV**

**ELECTRORECEPTION**

**CORE PROGRAM**

**SCIENTIFIC OFFICER: Dr. Igor Vodyanoy**

**BEGAN: OCTOBER 1, 1991**

**PROGRAM OBJECTIVE: TO UNDERSTAND HOW ELECTRICALLY-SENSITIVE FISH DETECT EXTREMELY WEAK ELECTRIC FIELDS IN THE NATURAL ENVIRONMENT.**

**NAVY OBJECTIVE: TO DEVELOP PRINCIPLES OF ELECTRORECEPTION APPLICABLE TO IMPROVED NAVY BIOSENSORS.**

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1905

R&T CODE: 4414922

PRINCIPAL INVESTIGATOR: Prof. Leon J. Bruner

INSTITUTION: University of California, Riverside, CA 92521-0413

GRANT TITLE: Circuit Models for Electrorception in Fish

REPORTING PERIOD: 1 August 1990 - 31 July 1991 (12 months)

AWARD PERIOD: 1 August 1990 - 31 July 1992

OBJECTIVE: To design, build, and test relaxation oscillator model circuits which can duplicate the known electrophysiological properties of the sensitive electroreceptor organs of marine elasmobranch fish; to demonstrate sensitivity of model circuit response comparable to that observed in behavioral studies on these fish; to use model studies as a guide to further electrophysiological studies.

ACCOMPLISHMENTS (last 12 months): The literature describes behavioral studies which have shown that marine elasmobranch fish are able to detect electric fields in their environment as weak as 5 nV/cm. Electrophysiological studies have identified the electroreceptor organs, the ampullae of Lorenzini, and have shown them to be in a tonic state characterized by repetitive electrical activity in the absence of stimulus. The resultant zero-stimulus discharge rate on associated afferent nerve fibers is ~ 40 imp/sec. This rate is modulated to higher or lower values depending upon the magnitude and polarity of applied stimulus. The resultant stimulus-response (S-R) curve has been measured in dissected specimens and reported in the literature.

Our work starts from the generally accepted viewpoint that oscillatory electrical activity in receptor cells of the ampullary epithelium drives the repetitive discharge on afferent nerve as described above. We model receptor cell activity using a relaxation oscillator, the basic circuit for which incorporates an operational amplifier, a tunnel diode having an I-V curve with a region of negative differential resistance, as well as resistors and a capacitor as linear passive elements. The oscillator circuit described in our original proposal, which we believe to be an original design, has been built and extensively tested during this reporting period. It traverses a well characterized limit cycle at a rate (frequency) in the physiological range which can be modulated by applied stimulus current (voltage) in the same manner as has been observed for electroreceptors. With appropriately chosen circuit parameters, our model exhibits an S-R curve which approximates those reported for electroreceptors. As a quantitative measure of sensitivity, we use the slope of the S-R curve at zero stimulus. Again a satisfactory match to electrophysiological data is obtained.

The neural coding scheme by which the animal evaluates electroreceptor output is most likely frequency coding, equivalent to a determination of the mean interspike period of the afferent nerve activity. This determination must be made in a time short enough to be of survival value to the animal, presumably 0.5 sec or less. Noise will introduce a period-to-period variation about the mean, characterizable as a period variance or standard deviation. This noise is expected to determine the minimum stimulus which the animal can detect. A shift of mean period by two standard deviations is a reasonable criterion for establishing the minimum detectable shift. The corresponding minimum detectable stimulus can then be inferred from the slope of the S-R curve, once the standard deviation in period is known. These considerations have led us to make an

analysis of the expected period noise properties of our model. The analysis is based upon general, circuit-independent properties of the relaxation oscillation process. Preliminary measurements generally support the predictions of the analysis.

SIGNIFICANCE: Our model studies indicate that a relaxation oscillation mechanism, operating within receptor cells of the ampullary epithelium, can account for the electrosensory capability of elasmobranch fish.

WORK PLAN: (next 12 months): While our relaxation oscillator model has already demonstrated the ability to replicate S-R data from the literature, a number of issues remain unresolved. The first relates to the fact that our model should properly be regarded as a 'single receptor cell' model, with all stimulus current flowing through this cell. In dissections of the electroreceptor organ, the afferent nerve is shredded into strands, only one of which is used to provide 'single unit' recording of nerve impulses likely to trace their origin to a single cell of the electrosensory epithelium. The stimulus current is, however, distributed over ~ 1,000 receptor cells of the epithelium in these experiments. It is therefore imperative that we explore the limits of sensitivity of our model, which have not been reached in experiments done to date.

The relevance of period noise measurements to receptor sensitivity determination has been mentioned above. To the best of our knowledge, no attempt has yet been made to perform such measurements on nerve fibers of the elasmobranch electroreceptor. Success in such measurements should enable us to determine whether short duration temporal averaging alone can suffice to account for sensitivity observed in behavioral studies on elasmobranchs, or whether spatial averaging over the entire receptor cell population of the ampulla must be invoked as well. This possibility for signal/noise enhancement has already been suggested in the literature.

We are planning a sabbatical leave for the first three months of 1992, during which time we expect to initiate a collaboration with Dr. A. J. Kalmijn of the Scripps Institute of Oceanography. At that time my graduate student, Mr. James R. Harvey, and I hope to make period noise measurements intended to shed light on the questions raised above.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Bruner, L. J. and Harvey, J. R. (1991) Circuit models for electroreception by fish. (Paper contributed to the 35th annual meeting of the Biophysical Society - abstract attached).
2. Bruner, L. J. and Harvey, J. R. A circuit model of the electroreceptive ampulla of Lorenzini. (Manuscript in preparation).



# ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1656

R&T CODE: 4414032

PRINCIPAL INVESTIGATOR: Dr. Watt W. Webb

INSTITUTION: Cornell University

GRANT TITLE: Molecular Mechanisms of Electric Field Interactions  
with Vertebrate Cells

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 January 1989 - 31 December 1992

OBJECTIVE: Our global objective is to gain understanding of the dynamics and molecular mechanisms of channel function particularly as involved in signal transduction.

ACCOMPLISHMENTS (last 12 months): Quantitative, reproducible single channel recordings of alamethicin channel activity with reproducible tension dependent records yield values for the area changes of the alamethicin channel in switching between adjacent conductance states. From this information, we have been able to develop new criteria for the modeling of alamethicin conductance state switching. Long, stable, low noise recordings produce statistically significant, high-frequency dwell time histograms. These timing histograms show that there are second, short-lived conformation states at each level that had not been seen before. We have estimated the current noise contribution of these brief, undetected, events and have shown that the contribution is 2-3 orders of magnitude below the measured current noise. Open channel noise measurements have detected two distinct classes of channels, a second class of duality which we have labeled persistent and non-persistent. These two classes have different open channel noise characteristics, conductance states and kinetic properties, but the molecular difference is not yet understood.

Kinetic analysis of channel switching has revealed new short lived open states of the alamethicin channel. Records of multiple conductance state events produce histograms of the probability distribution functions (pdf's) for the dwell times in each conductance level. Preliminary analysis suggests a linear superposition of two time constants that differ by several orders of magnitude. While the long time behavior for all transitions is clearly exponential, the short time behavior of the downward transitions can be fit equally well by a power law decay or the tail of an exponential.

SIGNIFICANCE: Tension activated ion channels are ubiquitous in a wide range of cell types, possibly serving as molecular mechano-electrical transducers of mechanical stimuli such as muscle extension, touch, sound vibrations, blood pressure, and osmotic stress. Elucidation of their physical mechanisms in cells is obscured by the cytoskeletal-membrane network. The reconstituted alamethicin channel presents an ideal model system in which to study the effect of membrane tension. This work has demonstrated the efficacy of lipid-protein interaction alone, without cytoskeletal interference, in affecting tension dependent ion channel behavior. In addition, we have demonstrated that membrane tension increases the partition of the alamethicin monomer into the membrane, suggesting that other proteins whose activity is dependent upon partitioning from the cytoplasm into the plasma membrane may also be sensitive to membrane tension.

WORK PLAN (next 12 months): To further distinguish between models for channel gating, we intend to continue to analyze the switching kinetics

of our single channel data. Our preliminary work showing that the dwell times can be represented by linear superpositions of two functions is consistent with either linear or parallel Markovian processes involving two states at each conductance level. Model-dependent rate constants can be obtained by substituting time constants into the solutions to the respective master equations. We also plan to attempt to obtain a large set of recordings of the non-persistent channel in order to characterize its kinetics.

We plan to develop a direct method for measuring changes in membrane area, thus allowing us to study tension effects of non-channel forming membrane proteins. A microinterferometer developed in our laboratory (3) will be used to measure the voltage dependent displacement of a hemispherical patch of membrane. The interferometer is sensitive to displacements to its 1 pm/Hz instrumental noise. For an 8  $\mu$  diameter hemispherical patch, the insertion of each 100  $\text{\AA}^2$  alamethicin monomer will cause a 0.02pm deflection of the membrane patch. For a flatter patch the sensitivity increases greatly but membrane stability may be a problem. The detectability of the signal depends on when the monomer inserts; if partition into the membrane is independent of channel switching, our ability to detect the voltage dependent area change would depend on the frequency of random inserts of 100 and would allow us to detect the membrane deflection with a signal/noise of 10 after averaging over  $\approx$ 100 voltage pulse events.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Opsahl, L.R., Mak, D.D., and Webb, W.W. (1990) Membrane tension selectivity of the alamethicin channel. Proc. Tenth International Biophysics Congress, p.398.
2. Mak, D.D. and Webb, W.W. (1991) Single-channel conductance noise in open alamethicin channels. Biophys. J. 59, 457a.
3. Denk, W. and Webb, W.W. (1990) Optical measurement of picometer displacements of transparent, microscopic objects. Appl. Opt. 29, 2382-91.

**MEMBRANE BIOLOGY I**

**MEMBRANE ELECTROCHEMISTRY**

**ACCELERATED RESEARCH INITIATIVE**

**SCIENTIFIC OFFICER: Dr. Igor Vodyanoy**

**BEGAN: OCTOBER 1, 1986**

**ENDS: SEPTEMBER 30, 1991**

**PROGRAM OBJECTIVE: TO DETERMINE THE PHYSICAL-CHEMICAL BASIS  
OF BIOLOGICAL CHARGE AND ENERGY TRANSDUCTION PROCESSES.**

**NAVY OBJECTIVE: TO OBTAIN INFORMATION ABOUT THE ELECTROCHEM-  
ICAL PROPERTIES OF MEMBRANE TRANSDUCTION PROTEINS TO ASSIST  
IN DESIGN OF FUTURE BIOSENSORS AND POWER SOURCES**

ANNUAL PROGRESS REPORT

GRANT #: N00014-91-J-1181

R&T CODE: 441k430

PRINCIPAL INVESTIGATOR: Dr. Martin Blank

INSTITUTION: Columbia University

GRANT TITLE: EFFECTS OF ALTERNATING CURRENTS ON Na,K-ATPase

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 October 1990 - 30 September 1992

OBJECTIVE: To study the effects of alternating currents on the Na,K-ATPase in order to elucidate the mechanisms of ion pumping and electromagnetic signal transduction by the enzyme.

ACCOMPLISHMENTS (last 12 months): We have established the conditions under which alternating currents (AC) increase or decrease the ATP splitting activity of the enzyme. The mechanism of both the enhancement and the inhibition by AC appears to involve an apparent increase in the binding of activating ions; increased binding can increase or decrease enzyme activity depending upon the ion concentrations. We have also shown a broad band dependence on frequency with an optimum at about 100 Hz, and have determined that the threshold for AC-inhibition at 100 Hz occurs at an electric field of about  $5 \times 10^{-6}$  V/cm with a corresponding current density of about 8 nA/cm<sup>2</sup>.

SIGNIFICANCE: Our experiments have corroborated and extended the studies by Tsong et al. on AC-enhanced ion transport via the Na,K-ATPase, and we have formulated a mechanism that appears to account for all observations. Furthermore, our measured threshold for AC-inhibition of the Na,K-ATPase is at the upper end of the electromagnetically induced electric fields producing changes in protein synthesis, indicating the effectiveness of weak signals. Our studies also suggest a mechanism for transmembrane signaling, as well as an explanation for the therapeutic effects of AC signals on damaged tissue that do not appear to affect normal tissue.

WORK PLAN (next 12 months): Since temperature affects ion activation, we shall use this to further test our mechanism for the effects of AC. We shall also study the effects of AC magnetic fields on the enzyme to see if the changes in ion activation are in line with the induced currents.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Milazzo, G., and Blank, M. (1990) Editors, "Bioelectrochemistry III: Charge Separation across Membranes", Plenum, New York, 337 pp.
2. Blank, M. and Soo, L. (1990) Ion Activation of the Na,K-ATPase. Bioelectrochem. Bioenerg. 24:51-61.
3. Blank, M. and Goodman, R. (1990) Charge Effects in Electromagnetic Stimulation of Biosynthesis. in "Bioelectrochemistry III: Charge Separation across Membranes", edited by G. Milazzo, and M. Blank, Plenum, New York, pp 311-324.
4. Blank, M. and Soo, L. (1990) The Effects of Alternating Currents on Na,K-ATPase Function. Abstract presented on 12 Jun 1990, Bioelectromag. Soc., p 40.
5. Blank, M. and Goodman, R. (1990) Electromagnetic Stimulation of Biosynthesis. Abstract presented on 11 Jun 1990, Bioelectromag. Soc., p 26.
6. Siskin, B.F., Blank, M. and Kurtz, W. (1990) Modification by PEMF of New Proteins Synthesized in Transected Rat Sciatic Nerve. Abstract presented on 13 Jun 1990, Bioelectromag. Soc., p 54.
7. Blank, M. and Soo, L. (1990) Ion Activation of the Na,K-ATPase. Abstract presented on 15 Oct 1990, BRAGS, p 15.
8. Blank, M. (1991) Membrane Transport: Insight from Colloid Science. in "Interfacial Phenomena in Biological Systems" edited by M. Bender. Dekker, New York, pp.337-366.
9. Blank, M. (1991) Extracellular and Cell Surface Effects of Electromagnetic Fields. in "Electromagnetics in Biology and Medicine", edited by C.T. Brighton and S.R. Pollack, San Francisco Press, in press.
10. Blank, M. and Soo, L. (1991) Ion Activation of Na,K-ATPase in Alternating Currents. in "Electromagnetics in Biology and Medicine", edited by C.T. Brighton and S.R. Pollack, San Francisco Press, in press.
11. Blank, M. and Soo, L. (1991) Changes in Ion Activation of the Na,K-ATPase in Alternating Currents. Abstract presented on 28 Feb 1991, Biophysical Soc., p 561a.
12. Blank, M. and Soo, L. (1991) Changes in Ion Activation at an Enzyme Surface due to Alternating Currents. Abstract 601 presented on 7 May 1991, Electrochem. Soc., p 155C.
13. Blank, M. Electrochemistry of Nerve Excitation, "Modern Aspects of Electrochemistry" (invited review in preparation).
14. Blank, M. and Vodyanoy, I. Editors, "Biomembrane Electrochemistry", Advances in Chemistry Series of the American Chemical Society Press, in press.
15. Blank, M. An Electrochemical Model for Voltage Gated Channels. submitted for publication in Advances in Chemistry.
16. Blank, M. and Soo, L. The Threshold for Alternating Current Inhibition of the Na,K-ATPase. submitted for publication in Bioelectromagnetics.
17. Blank, M. Na,K-ATPase Function in Alternating Currents. submitted for publication in the FASEB Journal.

## ANNUAL PROGRESS REPORT

Grant #: N00014-90-J-1024

R&T CODE: 441k816

PRINCIPAL INVESTIGATOR: Dr. Marco Colombini

INSTITUTION: University of Maryland at College Park

GRANT TITLE: Probing the Gating Mechanism of a Voltage Dependent Channel

REPORTING PERIOD: 16 June 1990 - 31 May 1991 (12 months)

AWARD Period: 1 October 1989 - 31 September 1991

OBJECTIVE: To gain molecular information about the structure and mechanism of voltage gating of the mitochondrial channel, VDAC. To understand how this protein functions and is controlled.

ACCOMPLISHMENTS (last 12 months): We succeeded in visualizing of the surface topography of both sides of two-dimensional crystals of VDAC formed in outer membranes from N. crassa. The surfaces look very similar: six pores surrounding an elevated region. The walls of the channels seem to be at or below the membrane surface. STEM was used to measure the mass per unit area of these crystals and the result indicates each channel is a monomer.

Having identified the transmembrane strands that form the wall of the open-state of the channel, we applied the same approach to the closed state. We found that charge changes at some locations that affected the selectivity of the open state, did not affect the selectivity of the closed state. Upon closure the strands in the center of the channel seem to remain fixed in place but those at either end move out, at least to some extent. At least some of the strands that seem to move out are part of the voltage sensor because charge changes at those locations result in the expected changes in voltage dependence. Attempts to test whether VDAC is a monomer or dimer by trying to generate hybrid channels (by examining single channels isolated from yeast cells containing both wild-type and mutant polypeptides) resulted in the observation of only normal wild-type and normal mutant channels indicating that VDAC is a monomer.

A soluble mitochondrial protein (called the VDAC modulator) induces VDAC to close at lower potentials was found in very different organisms (mammals, fungi, and plants). Remarkably, the modulator from one species could act on VDAC from all species tested. We further found that the VDAC modulator increased the voltage dependence of VDAC in ways similar to that observed for polyanions.

SIGNIFICANCE: We are achieving a detailed understanding of the molecular structure of VDAC and its gating mechanism.

WORK PLAN (next 12 months): The ability of the site-directed mutations to influence the voltage dependence of the channel should allow us to identify the portion of the protein that acts as a sensor. This work is underway but is a long and pains-taking process. These results will be integrated with the results of the selectivity experiments in order to understand the gating process. To help us with our understanding of how the channel behaves, we have fit our selectivity results to the fixed-charge-membrane theory of Teorell, with remarkable success. This will be tested further to see how successful this very simple theory actually is. We will also proceed with experiments designed to further test predictions arising from our developing understanding of VDAC's gating process. We plan to purify the VDAC modulator and use it to understand how VDAC is controlled.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Zhang, D. W. and Colombini, M. 1990. Group IIIA-Metal hydroxides indirectly neutralize the voltage sensor of the voltage-dependent mitochondrial channel, VDAC. Biochimica et Biophysica Acta, 1025: 127-134.
2. Liu, M. and Colombini, M. 1991. Voltage gating of the mitochondrial outer membrane channel VDAC is regulated by a very conserved protein. American Journal of Physiology, 260 (Cell Physiology 29): C371-C374.
3. Thomas, L., Kocsis, E., Colombini, M., Erbe, E., Trus, B.L. and Steven, A.C. 1991. Surface topography and molecular stoichiometry of the mitochondrial channel, VDAC, in crystalline arrays. Journal of Structural Biology, in press.
4. Wunder, U.R. and Colombini, M. 1991. Patch-clamping in liposomes containing whole mitochondrial membranes. Journal of Membrane Biology, (in press).
5. Thomas, L., Blachly-Dyson, E., Colombini, M. and Forte, M. 1991. Probing for the voltage sensor of the VDAC ion channel by site-directed mutagenesis. Biophys. J., 59:215a.
6. Liu, Mingyao and Colombini, M. 1991. The modulation of the mitochondrial outer membrane channel, VDAC, by a highly conserved protein from mitochondria. Biophys. J., 59: 93a.
7. Peng, Songzhi, Blachly-Dyson, E., Colombini, M. and Forte, M. 1991. Inferring the structural changes in VDAC, associated with the channel's gating process, through site-directed mutagenesis. Biophys. J., 59: 215a.
8. Colombini, M. 1991. Molecular insights into the structure and mode of action of VDAC. Biophys. J., 59: 117a.
9. Colombini, M., Peng, S., Blachly-Dyson, E. and Forte, M. 1991. Probing the Molecular Structure and Structural Changes of a Voltage-Gated Channel. In: Methods in Enzymology "Ion Channels" (Rudy, B. and Iverson, L.E., eds.) (in press)
10. Colombini, M. 1991. The mitochondrial channel, VDAC. In: Biomembrane Electrochemistry (Blank, M. and Vodyanoy, I. eds.) American Chemical Society, Washington D.C., (in press)

## ANNUAL PROGRESS REPORT

GRANT #: N000014-90-J-1137

R&T CODE: 441k705

PRINCIPAL INVESTIGATOR: Harvey M. Fishman, Ph.D.

INSTITUTION: University of Texas Medical Branch

GRANT TITLE: Gating kinetics and ion transfer in channels of nerve membrane

REPORTING PERIOD: 1 Nov 1990 - 1 Jun 1991 (7 months)

AWARD PERIOD: 1 Nov 1986 - 31 Oct 1991

OBJECTIVE: To obtain fundamental information on the gating and conductance properties of ion channels in nerve fibers and in axosomes (giant vesicles induced in axons).

ACCOMPLISHMENTS (last 7 months): 1. **Comparison of the  $K^+$ -conductance relaxation time  $\tau_n$ , in squid axon membrane determined by conventional Hodgkin-Huxley (HH) analysis versus direct linear analysis (admittance) analysis.** This work, which was begun in the initial year of this project, was completed and recently published in the journal Biophysical Chemistry. 2. **Ion channel studies in axosomes from transected squid giant axon.** a) **Rapid production of axosomes.** We developed a method for producing giant axosomes rapidly (in minutes). This method is an important advance because it yields axosomes consistently and rapidly so that patch clamping of axosomes can be done expeditiously and with relative ease. The method is described in an abstract presented at the 1990 Biophysical Society Meeting. b) **A  $Ca^{2+}$  channel in axosomes.** A  $Ca^{2+}$  channel was identified in excised patches of axosome produced as in (a) above. This work is also described in an abstract presented at the 1990 Biophysical Society Meeting. 3. **A new method for internally dialyzing, perfusing or injecting giant axons while observing them under high-magnification light microscopy.** To determine whether axosome formation occurs simply as a consequence of a rise in  $[Ca^{2+}]_i$ , axons were dialyzed while observing under high magnification ( $>200\times$ ) light microscopy. This work is described in an abstract presented at the 1991 Biophysical Society Meeting. **Induction of axosomes by internal dialysis with  $Ca^{2+}$ -containing solutions.** We could induce vesiculation by dialyzing axons with calcium solutions without fiber injury. This work is described in an abstract presented at the 1990 Society for Neuroscience Meeting.

SIGNIFICANCE: The comparison of kinetic parameter estimates from an HH analysis versus an admittance analysis indicates that an HH analysis cannot substitute for a direct linear analysis when comparing and relating macroscopic-derived



relaxation times to times derived from Markovian (linear) model fits of single channel currents. Rapid production of axosomes means that this will be an excellent system in which to study ion channels derived from intracellular organelles. The finding of a  $\text{Ca}^{2+}$  channel in axosomes suggests that the membrane origin of these vesicles is, in part, from intracellular organelles (e.g. endoplasmic reticulum). The new axon dialysis technique enables controlled introduction of a variety of substances (e.g., fluorescent dyes, enzymes and antibodies) internally in conjunction with light microscope methods that enable continuous observation of induced modifications in structures. Application of the dialysis technique showed that elevation of intracellular calcium level is, by itself, sufficient to induce vesiculation and axosome formation without neural injury.

WORK PLANS (next 12 months): The main focus will be on the internal dialysis of axons in order to develop our hypothesis that induced vesiculation is a manifestation of both short-term repair processes and degenerative processes. We wish to relate the former to growth cone formation and regeneration and the latter to neurodegenerative disease processes.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1702

R&T CODE: 441k181

PRINCIPAL INVESTIGATOR: Sol M. Gruner

INSTITUTION: Princeton University

GRANT TITLE: Lipid Dependent Mechanisms of Protein Pump Activity

REPORTING PERIOD: 1 June 1990 - 31 May 1991 (12 months)

AWARD PERIOD: 1 June 1990 -30 September 1991

OBJECTIVE: To investigate the hypothesis that membrane protein pump function is modulated by a *bioregulated* parameter called membrane lipid monolayer spontaneous curvature radius  $R_0$ , using high pressure techniques and lipid composition to vary  $R_0$ .

ACCOMPLISHMENTS (last 12 months): We have developed a high pressure X-ray diffraction system and measured structural changes when lipids in the  $H_{11}$  phase, which is the phase in which  $R_0$  can be directly assayed, are subjected to pressures of up to 3 kbar. We have for the first time succeeded in performing an electron density reconstruction of the  $H_{11}$  phase under pressure. We have been able to measure the much disputed volume changes that occur when a molecule of water is transferred from the bulk to the lipid-water mesophase [1]. The observed change for DOPE in the  $H_{11}$  phase is  $0.1\text{\AA}^3$  per water molecule transferred.

Studies on the effect of anesthetics like as *n*-alkanols on  $R_0$  [2] imply that  $R_0$  appears to be a colligative property. Furthermore, the effect of moderate pressures is found to reverse the effect of *n*-alkanols on  $R_0$ . An exciting consequence of this study is the idea that  $R_0$  may form the physical basis of the mechanism of anesthesia, which is generally thought to involve membrane channels [3].

Attempts to directly correlate the activity of  $\text{Ca}^{++}$  ATPase have been frustrated by difficulties with protein reconstitutions. We have discovered reconstituted vesicles exhibit a hitherto unreported protein-dependent ion-leakage which varies systematically with lipid composition of the reconstituted vesicles. We have concluded that this protein system is inappropriate for study of the correlation between lipid composition effects and protein activity and that lead literature in this field is artefactually based. Turning instead to the effects of pressure on protein pump activity, we find that the 'Temperature-Pressure equivalence' of the rate of decay of the reaction state *M* in the Bacteriorhodopsin has a value of 20 bar/k which is much more sensitive than most globular protein reactions. We have

found that the  $T - p$  equivalence of  $R_0$  is also precisely the same value. Preliminary experiments also indicate that photosynthetic reaction center from *Rhodopseudomonas Spheroides* is also highly sensitive to pressure.

SIGNIFICANCE: The remarkable coincidence of two temperature-pressure equivalences, one from our  $R_0$  work, and the other from a membrane protein suggest that pressure may be a very useful perturbative tool to explore our ideas of correlating  $R_0$  with protein activity without the need for extensive reconstitution procedures.

WORK PLAN (next 12 months): Lindblom et al. have reported the remarkable result that *Achoeloplasma laidlawii* appear to regulate the lipid composition in such a way as to maintain the onset temperature at which non-bilayer phases are formed. We have shown that other things being equal, this onset temperature is correlated with  $R_0$ . The bacterial lipids have been sent to us by Lindblom et al., and we have begun preliminary investigations to test the hypothesis that  $R_0$  is being homeostatically regulated.

In addition we have begun preliminary spectroscopic and kinetic investigations into the effects of high pressure on Bacteriorhodopsin and photo-synthetic reaction centers.

PUBLICATIONS AND ABSTRACTS (last 12 months):

[1] O. Narayan, P.T.C. So, D.C. Turner, M.W. Tate, S.M. Gruner and E. Shyamsunder "Volume constriction in lipid-water liquid crystal using high pressure X-ray diffraction" Phys. Rev. A **42** 7479 (1990).

[2] P.T.C. So, M.W. Tate, S.M. Gruner and E.M. Shyamsunder "X-ray diffraction studies on the effect of alkanols on DOPE-membranes under high pressure" Biophys. J. **57**:247a, 1990.

[3] Sol M. Gruner and Erramilli Shyamsunder "Is the mechanism of anesthesia related to lipid membrane spontaneous curvature?" N.Y. Acad. Sci. (in press).

## ANNUAL PROGRESS REPORT

Grant #: N00014-90-J-1960

R&T Code: 4414031

PRINCIPAL INVESTIGATOR: Dr. Andrew L. Harris

INSTITUTION: Johns Hopkins University

GRANT TITLE: Biophysics of Ion Channels That Span Two Bilayers

REPORTING PERIOD: 1 June 1990 - 31 May 1991 (12 months)

AWARD PERIOD: 1 June 1990 - 31 May 1993

OBJECTIVE: To understand the molecular biophysics of ion channels formed by connexin protein (the protein that forms gap junction channels between cells).

ACCOMPLISHMENTS (last 12 months): In the last year, we (1) completed the affinity-purification of connexin32, and (2) established that the affinity-purified connexin forms large, gated channels in single phospholipid membranes (liposomes and planar bilayers). The data strongly indicate that the channels are formed by single hemichannels, the subunits of the gap junction channel that each span a single cell membrane. Single-membrane connexin channels represent a form of the junctional channel accessible for detailed studies of permeation, gating modulation, and formation not possible *in situ*.

Connexin32 was affinity-purified from detergent-solubilized rat liver membranes using a monoclonal antibody. It is highly pure and not exposed to the denaturing conditions required for isolation of gap junction membranes. The connexin32 is predominantly in structures the size of single hemichannels (hexameric connexin). In unilamellar liposomes it induced permeability to sucrose and Lucifer Yellow. Low pH reversibly decreased the sucrose permeability. For a given amount of connexin, the number of sucrose-permeable liposomes is accounted for if a single hexamer can form a sucrose-permeable pore, but not if two hexamers are required. In bilayers, connexin32 produces conductances with asymmetric voltage sensitivity. Bilayer conductances show large fluctuations, clustering at intervals of 100-200pS. Some transitions are multiples of these values. Open channel currents have large, rapid fluctuations.

SIGNIFICANCE: Affinity-purified connexin32 forms channels in single membranes. Our system is well-defined and uniquely accessible for studies of detailed channel properties of a single connexin variety, including identification of factor(s) that directly modulate the channel and exploration of gating mechanisms. This is an essential step toward study of double-membrane connexin channels. The fact that the channels can open in single membrane suggests studies to identify the factor(s) that keep single hemichannels closed in plasma membranes. Identification of such a factor will be crucial for understanding many aspects of

junctional channel physiology and cell biology.

WORK PLAN: (next 12 months): The specific objective is to explore the properties of connexin channels in single and, if possible, double membranes. We will first describe the physiology of the single-membrane connexin channels that are in-hand. This will provide a groundwork of gating and permeation properties that is of intrinsic interest in channel biophysics and will be valuable for comparison with the properties of the double-membrane channels. These efforts will also help to define optimal conditions for the double-membrane reconstitution.

Using standard bilayer techniques, we will characterize the selectivity of the channels, with particular attention to the charge selectivity of molecules sufficiently large to interact with the walls of the pore. We will establish which putative physiological regulators affect the gating of the channels, and characterize the effect of each. We will characterize the voltage sensitivity, and test specific models for structural changes associated with gating will be tested. These studies will complete the basic characterization of gating and permeability of single-membrane connexin channels. We will explore several specific ways to achieve a double-membrane reconstitution of the connexin channels. If successful, we will explore the gating and permeability of the double-membrane channels.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Harris, A.L. (1991) Connexin32 forms ion channels in single artificial membranes. In: Biophysics of Gap Junction Channels, edited by C. Peracchia. Boca Raton: CRC Press, pp. 373-389.
2. Rhee, S.K. and Harris, A.L. (1991) Large channels formed by immunopurified connexin32 in single phospholipid membranes. Abstract presented 27 Feb 1991, Biophys. J. 59: 439a.
3. Harris, A.L., Rhee, S.K. and Bezrukov, S.M. (1991) Channels formed by immunopurified connexin32 in single membranes. Abstract presented 29 April 1991, ONR Membrane Electrochem. Mtg.
4. Harris, A.L., Walter, A., Paul, D., Goodenough, D.A. and Zimmerberg, J. Ion channels in single bilayers induced by rat connexin32. In press Proc. Natl. Acad. Sci. USA.
5. Rhee, S.K. and Harris, A.L. Affinity-purification of connexin32 using a monoclonal antibody. Accepted with minor revisions by Biochim. Biophys. Acta.
6. Rhee, S.K. and Harris, A.L. Channel-forming activity of immunoaffinity-purified connexin32 in single phospholipid membranes. Submitted to J. Biol. Chem.
8. Harris, A.L. Reconstitution of a protein that can form channels through two membranes. Submitted to Biomembrane Electrochemistry, edited by M. Blank and I. Vodyanoy. Washington, D.C.: Am. Chem. Soc.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1020

R&T CODE: 4414704

PRINCIPAL INVESTIGATOR: Huey W. Huang

INSTITUTION: Rice University

GRANT TITLE: Investigating the Structural Bases of Voltage-gating Model Channels by Using Aligned Multilayer Samples

REPORTING PERIOD: 1 June 1990 - 31 May 1991 (12 months)

AWARD PERIOD: 1 October 1989 - 30 September 1991

OBJECTIVE: To investigate the structural bases of the channeling properties of membrane active peptides, such as the ion selectivities and voltage dependence of alamethicin, gramicidin, and other synthetic channels, by x-ray diffraction and oriented circular dichroism.

ACCOMPLISHMENTS (last 12 months): By the method of oriented circular dichroism (OCD), we have discovered two different states of membrane-associated alamethicin. Depending on the type of lipid, the peptide-lipid ratio, and the chemical potential of water, alamethicin can either perpendicularly insert into the bilayer or bind parallel to the bilayer surface. Alamethicin switches between these two states reversibly (e.g., as the hydration of the sample changes) without changing its secondary structure. By x-ray diffraction, we have discovered the locations of the ion binding site in the gramicidin channel embedded in a bilayer. Two symmetric binding sites of  $Tl^+$  and of  $Ba^{++}$  are, respectively,  $9.6 \pm 0.3$  Å and  $13.0 \pm 0.2$  Å from the center of the channel.

SIGNIFICANCE: The OCD measurements resolved the controversy about the non-conducting state of alamethicin. The x-ray measurements resolved the controversy about the location of monovalent cation binding sites in the gramicidin channel; the positions are much closer to the center than previously expected. Both methods show the potential of application to many other structural problems of membrane proteins.

WORK PLAN (next 12 months): (1) Use x-ray and neutron diffractions to study the surface state and the inserted state of alamethicin that we discovered recently; (2) Use OCD to study the effect of electric field on alamethicin in multilayers; (3) Use x-ray diffraction and OCD to study the nonconducting state of gramicidin using synthetic variations which have the terminals modified so that they do not form dimers (collaboration with Roger Koeppe); (4) Use OCD and x-

ray diffraction to study the synthetic channels created by M. Montal (collaboration with Montal).

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Olah, G. A., Huang, H. W., Liu, W., and Wu, Y. (1991) Location of Ion Binding Sites in the Gramicidin Channel by X-ray Diffraction. J. Mol. Biol. 219
2. Liu, W., Teng, T. Y., Wu, Y., and Huang, H. W. (1991) Phase Determination for Membrane Diffraction by Anomalous Dispersion. to be published in Acta Cryst.
3. Huang, H. W. and Wu, Y. (1991) Alamethicin-Membrane Interactions and Voltage-gating Mechanism of Alamethicin Channel. to be published in Biophys. J.
4. Huang, H. W., Liu, W., Olah, G. A., and Wu, Y. (1991) Physical Techniques of Membrane Studies--Study of Membrane Protein Structures Using Multilayer Arrays. to be published in Progress in Surface Science.
5. Huang, H. W. (1991) Study of Channel-forming Peptides in Uniformly Aligned Multilayers of Membranes." in Membrane Electrochemistry, eds. M. Blank and I. Vodyanoy, American Chemical Society: Washington, DC.
6. Huang, H. W. (1990) Location of Ion Binding Sites in the Gramicidin Channel by X-ray Diffraction" 10th International Biophysics Congress Abstracts P5.5.3.
7. He, K., Huang, H. W., and Wu, Y. (1991) Gramicidin Channel by X-ray Diffraction. Biophys. J. 59, 318a.
8. Wu, Y. and Huang, H. W. (1991) Alamethicin-Membrane Interactions and Voltage-Gating Mechanism of Alamethicin Channel. Biophys. J. 59, 625a.
9. Huang, H. W. and Wu, Y. (1991) Free Energy of Alamethicin Channel Formation and Theory of Alamethicin Phase Transition. Biophys. J. 59, 625a.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1393

R&T CODE: 4414020

PRINCIPAL INVESTIGATOR: Garland R. Marshall, Ph.D.

INSTITUTION: Washington University

GRANT TITLE: Molecular Mechanism of Voltage-Dependent Ion Channels

REPORTING PERIOD: 1 June 1990 - 31 May 1991

AWARD PERIOD: 1 December 1989 - 30 November 1992

OBJECTIVE: *General:* To gain an understanding of the interaction of a molecule at the lipid/water interface of a membrane with the external electric field, using petaibols and the S4 segment of the sodium channel as a model system. *Specific:* To assess the role of the molecular dipole in sensing the imposed molecular field; the role of  $\alpha$ -helix- $3_{10}$ -helix transitions in responding to the imposed molecular field as the transduction mechanism; and the role of the channel mouth in determining the ion selectivity associated with channel opening.

### ACCOMPLISHMENTS (last 12 months):

Ac-(Lys(emerimicin 1-9))<sub>4</sub>-NH<sub>2</sub>NH<sub>2</sub>, tetraemerimicin, was prepared by solid phase coupling of the Boc-Lys(emerimicin 1-9) monomer as a probe of ion channel stoichiometry. The tetramer has been chemically characterized and its activity is currently being determined.

In order to determine length requirements for activity, a series of emerimicin analogs has been prepared in which the emerimicin 1-9 nonapeptide has been extended by the dipeptide, Ala-MeA-OBzl, to yield the following peptides: Ac-Phe-MeA-MeA-MeA-Leu-Gly-Val-MeA-MeA-Ala-MeA-OBzl (11 residues); Ac-Phe-MeA-MeA-Leu-Gly-Val-MeA-MeA-(Ala-MeA)<sub>2</sub>-OBzl (13 residues); and Ac-Phe-MeA-MeA-MeA-Leu-Gly-Val-MeA-MeA-(Ala-MeA)<sub>3</sub>-OBzl (15 residues). Our facilitated route to synthesis of the petaibols which combines solution and enzymatic couplings has been extended to alamethicin and the S-Eta<sup>12</sup> analogues of emerimicin III and IV. All new compounds have been submitted for assay on bilayers.

Solution NMR studies of emerimicins III and IV and their S-Eta<sup>12</sup> analogues in DMSO have been completed and suggest that all are helical. We have programmed a prototype software package which will allow quantitative evaluation of NOE data via relaxation rate matrix methods and should enable discrimination between  $\alpha$ ,  $3_{10}$ , and mixed helix types.

Two new solid state NMR techniques, TEDOR REDOR and double REDOR, have been calibrated using the emerimicin 1-9 nonapeptide, <sup>19</sup>FCH<sub>2</sub>CO-Phe-MeA-MeA-[<sup>13</sup>CO]MeA-[<sup>15</sup>N]Val-Gly-Leu-MeA-MeA-OBzl. The observed <sup>13</sup>C-<sup>15</sup>N internuclear distance, 7.8 and 7.9  $\pm$ 0.1 Å, respectively, is comparable to that found in the  $\alpha$ -helical crystal structure.

Umbrella sampling techniques have been used to model the  $\alpha$ -/ $3_{10}$ -helical transition in molecular dynamics simulations of MeA and Ala polymers. The results suggest that the energy barrier between the two forms is <1 Kcal/mol/residue and that the transition is populated by many structures of mixed helix type. Our earlier prediction, based on energy minimization studies, that the relative energy of the  $\alpha$  versus the  $3_{10}$  would be a function of length, was confirmed.

SIGNIFICANCE: Synthetic, spectroscopic, and computational tools will allow us to determine the stoichiometry and structure of the petaibol aggregates which function as ion channels and to develop models which describe conformational switches defining different channel states.

### WORK PLAN (next 12 months):



We will complete synthesis of alamethicin and emerimicin oligomers based on an oligolysine backbone, including tetramers, pentamers, and hexamers. In addition, we will complete, current synthetic efforts to use aza-crown-4 ethers as a scaffold for oligomers by coupling petaiabol fragments to  $\beta$ -alanines with which the ring nitrogens have been acylated.

Solution state NMR studies of emerimicin III and IV and C-terminal fragments will continue in deuterated micelles as well as other solvents of varying dielectric in order to determine the effect of the environment on observed helix type.

Solid state NMR studies will be extended to frozen solutions to confirm the results of solution NMR analyses, and to establish the utility of the technique in measuring distances in a heterogeneous lattice. TEDOR and double REDOR will be used to measure interatomic distances in emerimicin 1-9 and emerimicin III incorporated into lipid vesicles. Labelled analogues will be synthesized with  $^{13}\text{C}$ ,  $^{15}\text{N}$ , and  $^{19}\text{F}$  at appropriate positions, enabling us to determine the structure of these peptides in a biologically relevant context.

Molecular dynamics simulations will be done in a variety of solvent environments in order to determine solvent dielectric effect on the relative energetics of the  $\alpha$  and  $3_{10}$  helix and the transition between them.

#### PUBLICATIONS (last 12 months):

Hodgkin, E.E., J.D. Clark, K.R. Miller and G.R. Marshall. Conformational analysis and helical preferences of normal and  $\alpha,\alpha$ -dialkyl amino acids. *Biopolymers* 30:533-546 (1990).

Beusen, D.D., W.C. Hutton, J.J. Kotyk, J. Zabrocki, M.T. Leplawy and G.R. Marshall.  $^{13}\text{C}$  and  $^1\text{H}$  resonance assignments and structural determination of an emerimicin peptide DMSO. In: *Peptides 1990*, E. Giralt and D. Andreu, Eds., ESCOM, Leiden. pp. 545-547 (1991).

Leplawy, T., Jr., U. Slomczynska, M.T. Leplawy and G.R. Marshall. Peptides containing  $\alpha,\alpha$ -disubstituted amino acids. Experiments related to  $\alpha$ -methylcysteine. In: *Peptides 1990*, E. Giralt and D. Andreu, Eds., ESCOM, Leiden. pp. 285-286 (1991).

Slomczynska, U., J. Zabrocki, M.T. Leplawy and G.R. Marshall. Toward facilitated synthesis of petaiobols: Alamethicin via enzymatic segment condensation. In: *Peptides 1990*, E. Giralt and D. Andreu, Eds., ESCOM, Leiden. pp. 266-267 (1991).

Marshall, G.R., D.D. Beusen, J.D. Clark and E.E. Hodgkin. Helical transitions in peptides containing multiple  $\alpha,\alpha$ -dialkyl amino acids. In: *Protein: Structure, Dynamics, Design* V. Renugopalakrishna, P.R. Carey, I.C.P. Smith, S.-G. Huang, and A.C. Storer, Eds., ESCOM Science Publishers, Leiden. (1991), in press.

Clark, J.D., D.D. Beusen, E.E. Hodgkin and G.R. Marshall. Helical transitions in peptides. *Molecular Conformation and Biological Interactions* (Prof. G.N. Ramachandran Festschrift). P. Balaram and S. Ramaseshan, eds. Indian Academy of Sciences, Bangalore, India (1991), in press.

Marshall, G.R. and D.D. Beusen. The Structural Basis of Peptide Channel Formation. In *Biomembrane Electrochemistry*, M. Blank and I. Vodyanoy, eds., Adv. Chem., American Chemical Society, Washington, D.C. (in press).

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1469

R&T CODE: 4414911

PRINCIPAL INVESTIGATOR: Dr. Mauricio Montal

INSTITUTION: University of California, San Diego

GRANT TITLE: Channel Protein Engineering: A Novel Approach Towards the Molecular Dissection of Functional determinants in Ligand-regulated Channels

REPORTING PERIOD: 1 February 1990 - 31 January 1991 (12 months)

AWARD PERIOD: 1 February 1989 - 31 January 1992

OBJECTIVE: To identify the structures forming the channel lining of ligand-gated channels; to characterize the single channel properties of the pore-forming structure; and to model the structure using molecular mechanics and dynamics.

ACCOMPLISHMENTS: 1. **Molecular modeling of the pore forming structures of voltage-gated and ligand-gated channel proteins.** We pursued the modeling of the pore forming structures of the sodium channel and the acetylcholine receptor (Proteins 8:226-236, 1990). Further, we calculated the energy profiles for cations and found them to be consistent with the permeation properties observed experimentally. We introduced the use of "computer mutations" whereby specific residues are substituted and thereby various molecular contributions to the binding sites and energy barriers are deduced. Notably, for the acetylcholine 1 channel, an innermost site is found to be attributable to -OH groups of serine residues, consistent with our previous modeling and more recent experiments. However, a most external site is found to arise from a ring of highly conserved phenylalanine residues, not usually regarded as providing favorable interactions with ions. This site may provide favorable interactions with amphipathic channel blockers such as local anesthetics. Calculations are currently being extended to include a molecule of QX-222, a quaternary ammonium derivative of the local anesthetic lidocaine, within the lumen of the pore. Specific predictions for possible experimental mutations are made which can serve to test both the proposed structure, as well as the computational inferences we have drawn from it (Progr. Cell Res. 1:195-211, 1990). We are in the process of calculating the pore structure of the rat brain acetylcholine receptor  $\alpha_4$ , a neuronal receptor with presumed tetrameric structure. The M2 transmembrane segment may form the bundle that lines the pore: it shows extensive homology to the M2 segment of the muscle receptor, it has the same two

potential binding sites exposed to the lumen of the pore, namely a serine and a phenylalanine spaced eight residues apart and is also amphipathic. **2. Synthesis of tetrameric synthetic channel proteins and demonstration of channel blockage by a local anesthetic.** We have extended our synporin strategy (Proc. Natl. Acad. Sci. USA 87:6929-6933, 1990) to design and synthesize pore proteins based on sequence information and oligomeric number. This concerted approach encompassing the technologies of molecular modeling, synthetic chemistry and single channel recordings in lipid bilayers is proving to be valuable and identifying and further examining the functional pore entities of an entire channel protein (FASEB J. 4:2623-265, 1990). Thus far, we have designed and synthesized four homotetrameric proteins which imitate the sequence of a highly conserved segment predicted to line the pore of channel proteins. A. For the nicotinic acetylcholine recaptor from *Torpedo californica* a muscle-like receptor, the sequence is: EKMSTAISVLLAQAFLLLTSQR; B,C. For the nicotinic acetylcholine receptor from brain, a neuronal acetylcholine recaptor, the sequence for the  $\alpha_3$  or  $\alpha_4$  subunit is: EKVTLCISVLLSTVFLLLITE; and for the  $\beta$  subunit is: EKVTLAISVLLALTVFLLLISK. Where A is replacing the authentic cysteine in this position. As control proteins for the first three cholinergic recaptor channels, we synthesized a similar tethered tetramer with M1 peptides. None of the control proteins form channels in lipid bilayers, in accord with expectations. The synthetic proteins emulate the pore properties of the authentic channels, including single channel conductance, cation selectivity, transitions between closed and open states in the millisecond time range and sensitivity to channel blockers. In membranes composed of phosphatidylethanolamine, the single channel conductance in symmetric 0.5M KCl or 0.5 NaCl of the muscle-like channel protein, is 20 pS and 15 pS, respectively. For the neuronal-like channel protein, the corresponding values are 28 pS and 14 pS respectively. The apparent  $K_d$  for channel blocking by QX-222 in 0.5 KCl is  $\sim 10^{-6}$ M. Thus, the M2 synthetic channel proteins exhibit the sensitivity to local blockers characteristic of the authentic cholinergic receptor.

**SIGNIFICANCE:** The current excitement is based on the availability of the structure model and of the synthetic pore protein, which should prove valuable in assessing the functional role of key residues and should facilitate the conceptual design of drugs that alter the pore by blocking it from the aqueous pathways or via the hydrophobic access to the protein from the bilayer interface.

**WORK PLAN:** We are in the process of examining the key role of specific residues in determining the activity of these channel proteins and their sensitivity to local anesthetic

blockers by designing and synthesizing analogues where such residues are substituted. These studies are guided by the results of the "computer mutations" previously described.

#### PUBLICATIONS AND ABSTRACTS:

1. Montal, M., M.S. Montal and J. Tomich. Synporins: Synthetic proteins that emulate the pore structure of biological ionic channels. *Proc. Natl. Acad. Sci. USA*, 87:6929-6933 (1990).
2. Montal, M. Molecular anatomy and molecular design of channel proteins. *FASEB J.*, 4:2623-2635 (1990).
3. Oiki, S., V.E. Madison and M. Montal Bundles of amphipathic trans-membrane  $\alpha$ -helices as a structural motif for ion-conducting channel proteins: Studies on sodium channels and acetylcholine receptors. In: *PROTEINS: Structure, Function and Genetics.*, 8:226-236 (1990).
4. Bechinger, B., Kim, Y., Chirlian, L.E., Gisell, J., Neumann, J.-M., Montal, M., Tomich, J., Zasloff, M. and Opella, S.J. Orientations of amphipathic helical peptides in membrane bilayers determined by solid-state NMR spectroscopy. *J. Biomol. NMR* in press.
5. Tomich, J.M. Grove, A., Iwamoto, T., Marrer, S., Montal, S.M., and Montal, M. Design principles and chemical synthesis of oligomeric channel proteins. In: *Membrane Electrochemistry*. I. Vodyanoy and M. Blank, ed. American Chemical society, ACS publishing, in press. (1991).
6. Grove, A., Tomich, J.M. and Montal, M. A molecular blueprint for the pore-forming structure of voltage-gated calcium channels. *Proc. Natl. Acad. Sci. USA* In press. (1991).
7. Grove, A., Iwamoto, T., Montal, M.S., Tomich, J.M., and Montal, M. Synthetic peptides and proteins as models for the pore-forming structure of channel proteins. In: *Methods in Enzymology: Ion Channels*. B. Rudy and L.E. Iverson, ed(s). Academic Press, New York, In press.. (1991)

## ANNUAL PROGRESS REPORT

GRANT NO0014-89-J-1792  
441k707

R&T CODE:

PRINCIPAL INVESTIGATOR: Dr. David S. Perlin

INSTITUTION: The Public Health Research Institute

GRANT TITLE: Mechanism of H<sup>+</sup>-Transport and Membrane Voltage  
Interactions in a Yeast H<sup>+</sup>-ATPase

REPORTING PERIOD: 1 June 1990 - 31 May 1991 (12 months)

AWARD PERIOD: 1 June 1989 - 31 May 1991

OBJECTIVE: To identify protein structure domains of the yeast plasma membrane H<sup>+</sup>-ATPase that participate in proton transport, membrane voltage interactions and energy coupling by examining normal and mutant forms of the enzyme.

ACCOMPLISHMENTS (last 12 months): In the second year of this two year project, short and long-range protein-protein interactions within and between the catalytic ATPase and translocation domains were explored by a detailed revertant analysis. Random second site mutations were generated to complement a primary site mutation (F368) within the catalytic center that alters both ATP hydrolysis and electrogenic proton transport. Complementing second site mutations were found within the catalytic center and in an adjacent hydrophilic loop region that has been proposed to interact with the catalytic site. In addition, many of the second site mutations mapped to five of eight putative transmembrane segments (1,2,3,4 and 7). Transmembrane segments 1 and 2 show a high mutation frequency and appear to be highly conformationally active. All mutations in this region were found to alter the electrogenic properties of the enzyme. This region has been modeled as a helical hairpin. The region around the closed loop was mutagenized by site-directed mutagenesis and found to greatly influence electrogenic proton transport and ATP hydrolysis. A second revertant analysis was performed to identify complementing second site mutations to a strong A135V mutation near the top of the loop region that alters both electrogenic proton transport and ATP hydrolysis. In this case, only mutations within other transmembrane sectors were found. This analysis has helped molecular modeling studies intended to develop a 3-dimensional view of the membrane sector portion of the enzyme.

SIGNIFICANCE: It is our assertion that transmembrane sectors 1 and 2 participate in electrogenic proton transport and influence the ATP hydrolytic domain through a coupling

mechanism that may involve the polar loop extension between transmembrane sectors 2 and 3.

WORK PLAN (next 12 months): The research objectives of the final year will be to: 1) probe by site-directed and random-localized mutagenesis the importance of amino acids comprising transmembrane helices 1 and 2 in electrogenic proton transport, 2) use mutant analyses to extend and refine molecular modeling studies intended to define interactions between protein structure elements and 3) test models by directed mutagenesis. The goal of this work will be to determine the role of transmembrane segments 1 and 2 in coupled electrogenic proton transport and place these elements within the context of an overall protein structure model.

The foundation of our approach continues to be an analysis of mutant enzymes with defects in electrogenic proton translocation. Site-directed mutagenesis has proved to be a valuable, albeit limited, approach for analyzing the role of specific amino acid residues. A new emphasis has been placed on isolating complementing second-site mutations which complement a primary site defect. This approach has proved more efficient and productive for the analysis of functional protein structure domains. Finally, molecular modeling is being used as an approach to visualize membrane structure to circumvent problems associated with crystallization of the H<sup>+</sup>-ATPase.

PUBLICATIONS AND ABSTRACTS (last 12 months):

1. Seto-Young, D. and Perlin, D.S. (1990) Effect of Membrane Voltage on the Plasma Membrane H<sup>+</sup>-ATPase of Saccharomyces cerevisia. J. Biol. Chem. 266, 1383-1389
2. Harris, S.L., Na, S., Haber, J.E., Seto-Young, D., Monk, B.C. and Perlin, D.S. 1991 Probing protein dynamics in a yeast H<sup>+</sup>-ATPase. Biophysical J. 59, 562a
3. Perlin, D.S., Seto-Young, D., Monk, B.C., Harris, S.J., Na, S. and Haber, J.E. 1990 Genetic approaches to electrogenic proton transport by a yeast H<sup>+</sup>-ATPase. Submitted to the American Chemical Society Books.
4. Monk, B.C., Feng, W., Harris, S.L., Na, S., Haber, J.E. and Perlin, D.S. 1991 Modeling of the mebrane sector of the fungal plasma membrane proton pump. Submitted to Proteins.
5. Harris, S.L., Perlin, D.S., Seto-Young, D. and Haber, J.E. 1991 Evidence for coupling between membrane and cytoplasmic domains of the yeast plasma membrane H<sup>+</sup>-ATPase. An analysis of intragenic revertants of *pma1-105*. Submitted to the J. Biol. Chem.

GRANT #: N00014-89-J-3190

R&T CODE:441k903

PRINCIPAL INVESTIGATOR: Michael J. Wilcox, Ph.D.

INSTITUTION: University of New Mexico School of Medicine

GRANT TITLE: Electrochemistry of Channels Coupling  
Photoreceptor Axon Terminals in the Eye of the Fly

REPORTING PERIOD: 1 August 90 - 1 June 91

AWARD PERIOD: 1 August 89 - 31 July 92

OBJECTIVE: To investigate the control mechanism and degree of both electrical and dye coupling between the six photoreceptor axon terminals, whose rhabdomeres share a common visual axis in the neuro ommatidial arrangement of the fly's compound eye.

ACCOMPLISHMENTS: DYE COUPLING: Formerly dyes were injected into photoreceptor somas. No dye coupling was observed. The hypothesis that the axon terminal did not readily fill was tested by injecting fluorescent dyes directly into the axon terminal. Enhanced sensitivity of an in vivo preparation allows small amounts of dye in neighboring cells. Dyes of molecular weight 132 to 547, were injected alone or coinjected with the calcium chelating agent EGTA and/or high concentrations of KOH. Sequestration of calcium or raising the intracellular pH opens closed gap junctions in oocytes, mature fibroblasts, corneal and pigment epithelium and embryonic mesoderm and epidermis. No coupling was found.

OPTICAL MANIPULATION: A confocal microscope was built and modified to independently stimulate single photoreceptor rhabdomeres that share the same visual axis within a single neuro-ommatidium. A HeCd laser was spatially filtered and expanded to a diameter of 1 mm. The beam was split to form two pathways that illuminate pinholes 600  $\mu\text{m}$  in diameter. These pinholes are imaged at the back focal plane of the objective, a 25mm achromat lens. The two beams are made coaxial using uncoated pellicles. Thus, two independent beams are imaged at infinity, along a single axis subtending 0.2° of arc to the fly's eye. Independently moveable field diaphragms, formed by 100  $\mu\text{m}$  diameter pinholes, are imaged onto individual facet lenses of the fly's compound eye, allowing stimulation of only one cell in the ommatidium.

ELECTRICAL COUPLING: Using this imaging capability the degree of electrical coupling of the axon terminals can be determined. We measured this degree of coupling to be as high as 80-93% in adjacent axon terminals and this same degree of coupling is present throughout the linear range of voltage response for the photoreceptor. The surprising finding was that sequential stimulation of two coupled axon terminals with stimuli balanced to give equal excitation of the impaled cell showed nonlinear summation.

**SIGNIFICANCE:** Lack of dye coupling could be due to restriction in the pore of neural gap junctions, however, other dipteran neurons are electrically and dye coupled. Morphological measurements from freeze fracture replicas of gap junctions in fly photoreceptor axon terminals show that the pore has a diameter of 9-11 nm, more than 5X the minimum diameter of Rhodamine B. These specialized junctions could have a restriction within the pore but this is unlikely in view of the highly conserved amino acid sequence of gap junctions from several species. Another possibility is the fuzzy zone seen adjacent to neural gap junctions, thought to confer calcium sensitivity and perhaps voltage sensitivity and rectification to neural gap junctions. If coupling via gap junctions were purely resistive, coupling should be linear. Nonlinear summation suggests that at the earliest opportunity for interaction of neural signals (presynaptic summation) the signal is already nonlinear. Nonlinearity is essential for processes like motion detection. Such interactions were previously thought to occur only in higher visual processing. For technical reasons, this type analysis was never applied to this level of the retina. Significance of the small angular subtense of the stimulus is that it is less than one tenth the theoretical limit of resolution of the fly's eye. Showing detectability of either sequential stimulation or displacement of the stimulus by less than the spacing of the receptor matrix, will prove the capability of the retinal circuitry for hyperacuity.

**WORK PLAN** (next 12 months): A three pronged attack on the mechanism of electrical and dye coupling affects the channel protein itself, the adjacent fuzzy area and control molecules. The sequence of conventional gap junctions is highly conserved in the transmembrane, N-terminus and intermediate regions. The insertion region is highly HS rich. Histochemical analysis of the terminal will begin with oxidation of disulfide bonds to induce terminal decoupling. The intermediate zone of the gap junction molecule is selectively digested by chymotrypsin. Current models of the molecule attribute control of channel opening and closing to this region of the molecule. Intraterminal injection of dye and chymotrypsin will be used to induce dye coupling and change electrical coupling, measured by sequential stimulation of neighboring cells. Calcium-calmodulin interaction with gap junctions will be modified by treatment with trifluoperazine and pharmacological manipulation via extracellular space and intraterminal injection of lanthanum, ruthenium red and vanadate. Interaction between cytoskeleton and gap junctions will be monitored by electrical recording while injecting cytochalasin B and calcium-saturated EGTA along with dye.

**PUBLICATIONS AND ABSTRACTS** (last 12 months):

Sexually dimorphic axon terminals show plasticity of form and function. Submitted to the Journal of Neuroscience.



**MEMBRANE BIOLOGY II**

**WATER AT BIOLOGICAL INTERFACES**

**CORE PROGRAM**

**SCIENTIFIC OFFICERS: Drs. Harold Bright and Igor Vodyanoy**

**BEGAN: OCTOBER 1, 1990**

**PROGRAM OBJECTIVE: TO UNDERSTAND THE NATURE OF WATER INTER-ACTIONS AT BIOLOGICAL INTERFACES**

**NAVY OBJECTIVE: TO PROVIDE FUNDAMENTAL KNOWLEDGE ABOUT WATER-BIOLOGICAL INTERFACES TO USE IN BIOTECHNOLOGY AND MEDICINE.**

## ANNUAL PROGRESS REPORT

Grant #: N00014-89-J-3002

R&T CODE: 4414912

PRINCIPAL INVESTIGATOR: Max Berkowitz

INSTITUTION: University of North Carolina at Chapel Hill

GRANT TITLE: Structure and Dynamics of Aqueous Solutions Next to and Between Membrane Surfaces.

REPORTING PERIOD: July 1990-May 1991

AWARD PERIOD: 6/1/89-5/31/92

OBJECTIVE: To investigate Structural and dynamical properties of aqueous solutions next to and between surfaces of biological macromolecules, particularly membranes.

ACCOMPLISHMENTS (last 12 months): In order to understand the origin of the hydration force acting between layers of phospholipid molecules we have performed a molecular dynamics computer simulation on a system composed of water molecules embedded between surfaces of DLPE molecules. In our first simulation the distance between membrane surfaces is 1.6 nm. The simulation shows that the orientational polarization of water displays the oscillatory decay which is independent of thermal motion of the head group of the membrane molecules. Since the Marcelja-Radic theory predicts a monotonic decay for the order parameter we conclude that the orientational polarization is not a good order parameter. The dynamical properties of water are also investigated. We observe the increase in the diffusion coefficient for water in the middle of layer between the membranes. This is related to the small decrease in the number of hydrogen bonds in water. A second simulation, where the distance between the membrane surfaces is smaller is already completed and the analysis of this simulation is now in progress.

SIGNIFICANCE: Our simulation is the first simulation where the molecular details of the flexible membrane surface is taken into account. We also proved without any doubts that the flexibility of the head groups of phospholipid molecules does not cause the change in the water polarization.

WORK PLAN (next 12 months): We are presently analyzing the data from our second DLPE/water simulation, which was performed at shorter interbilayer distance. The results from this simulation will be compared with the results from our previous simulation performed at larger interbilayer separation. We also plan to replace DLPE head groups with the DLPC groups and see how this change will influence the behavior of water. We will examine the changes in hydrogen bonding pattern and see if hydrogen bonding can be connected

with the order parameter concept. We also plan to connect our results with recent more advanced theories of hydration force.

PUBLICATIONS AND ABSTRACTS (last 12 monts):

1. M. Berkowitz and K. Raghavan (1991) Computer simulation of a water/membrane interface. Langmuir (in press)
2. M. Berkowitz and K. Raghavan (1991) Electrostatics of a membrane/water interface. A chapter in ACS book on Biomembrane Electrochemistry, editors M. Blank and I. Vodyanoy

## ANNUAL PROGRESS REPORT

GRANT #: N00014-89-J-1622

R&T CODE: 4414904

PRINCIPAL INVESTIGATOR: Manachem Gutman

INSTITUTION: Tel Aviv University

GRANT TITLE: Probing of membranes surface by dynamic measurements of proton diffusion

REPORTING PERIOD: 1 May 1990 - 1 May 1991

AWARD PERIOD: 1 February 1989 - 1 February 1992

OBJECTIVE: To carry out dynamic measurements of proton diffusion within the thin water layers as found in bioenergetic organelles.

To analyze the observed dynamics according to a comprehensive biophysical model and to derive from his analysis how the geometry of the reaction space determines the physical properties of the microenvironment.

ACCOMPLISHMENTS (last 12 months): The mathematical model of N. Agmon, for the study of geminate recombination in spherical homogeneous space (Pines et al., J. Chem. Phys. 88: 5620, 1988) was converted to describe the proton-anion interaction in the mesoscopic space between phospholipid membranes. Time resolved measurements of proton-anion recombination were carried out with three phosphatidyl choline preparations under varying osmotic pressure.

Analysis of the results indicates that the diffusion coefficient of the proton is independent of the width of the aqueous layer. The same value, that of bulk water, was measured even when the layer was as thin as 3 molecules across. We conclude that the ordering of water in the intermembranal space is too small to modify the propagation of the proton.

The effective dielectric constant of the thin water layer between lipid membranes is  $\epsilon_{eff}=40-50$ . This value controls the intensity of electrostatic interactions in all energy conserving organelles where oxidative and photosynthetic phosphorylation takes place.

The lateral compression of phosphocholine headgroup reduces the accessibility of the proton to the phospho moiety of the lipid. This indicates that the headgroup region is flexible and exhibits delicate conformational changes.

SIGNIFICANCE: The methodology we introduced (experimental plus theoretical analysis) revealed new, quantitative information on physical condition between the lipid membranes. These measurements increase our ability to draw a general structure/function relationship between the geometric features of mesoscopic size cavities and their biological functions.

WORK PLAN: In the coming year (if the project is continued), we shall study the shape-physical properties correlation in a new class of cavities - the "infinitely" long aqueous tubes of the reversed phase hexagonal ( $H_{II}$ ) lipid-water system. These studies will be extended to natural pores like the anionic channel PhoE porine of E. coli.

In a parallel effort we shall monitor the protonation dynamics of the native mitochondrial membrane and investigate the redox dynamics driven by this perturbation. The equipment for this experiment, a coherent dye laser, has been acquired with the ONR's partial support.

## PUBLICATIONS:

### a) Refereed journals

1. M. Gutman and E. Nachliel (1990) The dynamic aspects of proton transfer processes. *Biochim. Biophys. Acta* 1015: 391-414.
2. S. Rochel, E. Nachliel, D. Huppert and M. Gutman (1990) Proton dissociation dynamics in the aqueous layer of multilamellar phospholipid vesicles. *J. Membrane Biol.* 118: 225-232.
3. R. Yam, E. Nachliel, S. Kiriyati, M. Gutman and D. Huppert (1991) Proton transfer dynamics in the non homogeneous electric field of a protein. *Biophys. J.* 59: 4-11.

### b) Chapters in books

1. R. Yam, S. Kiriyati, E. Nachliel, and M. Guman. Evaluation of protein's electric field by dynamic measurements of proton transfer. In: *Electrified interfaces in Physics, Chemistry, and Biology*. R. Guidell, ed. NATO ASI, Kluwer Academic Publishers, Dordrecht, Holland, in press.
- 2) M. Gutman, E. Shimoni, and Y. Tsfadia. Diffusion of proton in microscopic space: effect of geometric constraints and dielectric discontinuities. In: *Electron and proton transfer in Chemistry and Biology*. A. Miller, ed. Elsevier, Amsterdam, in press.
- 3) M. gutman. Time resolved dynamics of proton diffusion at a water-membrane interface. I. Vodyanoy and M. Blank, eds. ACS Books, in press.

## LECTURES (INVITED SPEAKER)

1. Electrified interfaces. NATO ASI, Varenna, Italy, July 1990.
2. Proton-electron transfer. ZIF, Bielefeld, Germany, September 1990.
3. Gordon Research Conference on Protons and Membranes, february 1991.
4. Israel Biochemical Society, April 1991.
5. Contractor Meeting, ONR, Airlie, USA, May 1991.

Annual Progress Report

ONR Grant # N00014-91-F-0201

R & T Code 441k701

Principal Investigator: V. Adrian Parsegian

Institution: National Institutes of Health

Grant Title: Electrochemical Properties of Ionic Proteins and Other Proteins with Aqueous Cavities.

Reporting Period: 06/01/90 - 05/31/91

Award Period: 10/01/90 - 09/30/93

**OBJECTIVE:** To measure changes in the structure of functioning proteins by methods developed to determine forces between macromolecules or membranes; to connect emergent principles of molecular interaction with the control of protein function.

**ACCOMPLISHMENTS (last 12 months):**

Using osmotic stress, we have found that some 60 water molecules go on to hemoglobin when it switches between the "tense" deoxy and the "relaxed" oxy forms. It appears that water should be treated as a liganding species much as oxygen or other allosteric effectors.

From the temperature dependence of hydration forces measured between Mn-DNA double helices, we have been able to extract the Entropy and the Enthalpy of interaction between these molecules. The Entropy of bringing Mn-DNA molecules together is positive and appears to be due to the release of ordered water from the vicinity of the macromolecule itself or the bound Mn.

The alpha-toxin trans-membrane channel from *S. Aureus* has been reconstituted into bilayer membranes and osmotically stressed to provide the information that about 700 water molecules enter and leave this channel when it opens and closes under applied voltage. This channel appears also to have different pH sensitivity from each side of the membrane.

**SIGNIFICANCE:** Hydration, seen as a structural force between molecules approaching contact or as a factor in determining states of proteins, is a significant element in macromolecular stability. Not only the opening and closing of ion channels but the transitions between allosteric states should be viewed as events that incur potentially large (and now measurable) changes in associated water solvent.

**WORK PLAN (next 12 months):**

We will apply osmotic stress to several proteins -- hemoglobin, certain enzymes, ionic channels -- to gauge further the importance of the change in solvation for controlling transitions between functioning states. It is particularly important now to learn to distinguish the direct action of a ligand, the binding of an anion to hemoglobin, for example, from the indirect action of that same substance on molecular hydration. (Salt will bind as well as change water activity.) Until now, such distinction has been completely, perhaps crucially, missing in studies of allosterism. We will work with mixtures of salt and neutral, non-binding, solutes to control water and ligand activities separately.

We plan to examine the importance of changes in the protein surface by applying osmotic stress to several different hemoglobin mutants.

We will investigate the sources of hydration forces, through osmotic stress/temperature measurements as well as theoretical models of attractive and repulsive structural forces. It now appears possible also to measure the change in the density of Mn-DNA suspensions that are assembling under the action of attractive hydration forces; density measurements combined with osmotic stress may allow us to identify the sites of ion binding and of the release of structured water.

We hope to complete internal aqueous volume measurements for one or two more channels. Together with these measurements we will begin a theoretical analysis of water in cylinder-like cavities to explore the importance of shape on solvation.

#### PUBLICATIONS AND ABSTRACTS (last 12 months)

1. Zimmerberg, J., Bezanilla, F., Parsegian, V.A. (1990). Solute inaccessible volume changes during opening of the potassium channel of the squid giant axon. *Biophys. J.* 57: 1049-1064.
2. Rau, D.C., Parsegian, V.A. (1990). Direct measurement of forces between linear polysaccharides xanthan and schizophyllan. *Science* 249: 1278-1281.
3. Parsegian, V.A., Rand, R.P., Fuller, M.L., (1991). Direct osmotic stress measurements of hydration and electrostatic double layer forces between bilayers of double-chained ammonium acetate surfactants. *J. Phys. Chem.* (in press).
4. Parsegian, V.A., Rand, R.P., (1991). On the unlikelihood of molecular protrusion as the source of hydration forces. *Langmuir* (in press).
5. Podgornik, R., Parsegian, V.A. (1991). An electrostatic-surface stability interpretation of the "hydrophobic" force inferred to occur between mica plates in solutions of soluble surfactants. *Chemical Physics* (in press).
6. Leikin, S., Kornyshev, A.A. (1991). Mean-field theory of dehydration transitions. *Phys. Rev. A* (in press).

#### MANUSCRIPTS SUBMITTED

7. Leikin, S., Rau, D.C., Parsegian, V.A. (1991). Measured entropy of water between DNA double helices as a function of separation. *Phys. Rev. A.* (submitted).
8. Rau, D.C., Parsegian, V.A. (1991). Direct Measurement of Temperature - Dependent Solvation forces between DNA double helices. *Biophys. J.* (submitted).

#### ABSTRACTS

Leikin, S., Kornyshev, A.A. (1991). New results in theory of hydration forces: The role of surface structure of lipid membranes, dehydration transition. *Biophys. J.* 59: 547a.

Kasianowicz, J.J., Moore, C.R., Zimmerberg, J., Pasternak, C., Bashford, C.L., Parsegian, V.A. (1991). Two-sided protonation of the alpha-toxin channel inferred from the effects of trans-membrane Ph gradients. *Biophys. J.* 59: 458a.

Colombo, M.F., Rau, D.C., Parsegian, V.A. (1991). Regulation of hemoglobin activity by water molecules. *Biophys. J.* 59: 611a.

**SINGLE NEURON COMPUTATION**

**ACCELERATED RESEARCH INITIATIVE**

**SCIENTIFIC OFFICER: Drs. Thomas McKenna and Igor Vodyanoy**

**BEGINS: OCTOBER 1, 1992**

**ENDS: SEPTEMBER 30, 1997**

**PROGRAM OBJECTIVE: TO CONDUCT INTERDISCIPLINARY BIOPHYSICAL AND MODELLING INVESTIGATIONS OF NEURONS IN ORDER TO FORMALIZE THE COMPUTATIONAL MECHANISMS PERFORMED BY NEURONS.**

**NAVY OBJECTIVE: TO PRODUCE ENHANCED SIGNAL PROCESSING ELEMENTS.**



# ANNUAL PROGRESS REPORT

GRANT #: N00014- 91-J-1345

R&T CODE: 4414139

PRINCIPLE INVESTIGATOR: Prof. Arnold J. Mandell

INSTITUTION: Florida Atlantic University

GRANT TITLE: Intermittency and Coding in the Diffuse Brain Systems

REPORTING PERIOD: 1 JUNE 1990 - 31 MAY 1991

AWARD PERIOD: 1 January, 1991 - 31 December, 1991

OBJECTIVE: To develop quantitative descriptions and models of intermittency (bursting) patterns, quasiperiodic driving and resonances, and strange *nonchaotic* attractors in single neuron and global electrophysiological phenomena in brain using time series of real data, analog and digital computer simulation, and abstract dynamical systems and measure theory.

ACCOMPLISHMENTS (last twelve months): In the context of the Single Neuron Computation Program, we are using time series of interspike intervals from single neuron recordings from the locus coeruleus (norepinephrine), ventral tegmental A10 region (dopamine), dorsal raphe nucleus (serotonin), dorsal raphe (reticular formation nuclei), and gigantocellularis nuclei (reticular formation) to study trans-thalamic and extrathalamic influences on global brain behavior including EEG and rat behavior. We have begun to relate this data to the bursting behavior of brain biogenic amine biosynthetic enzyme behavior which regulate single neuron chemical control of autoreceptor dynamics. Our data sources are on computer disc and come from the laboratories of Bloom, Carlson, Foote, Vertes, Kreuger, Knapp, Russo, Kelso, DeLuca, McElroy, Guillemin, Brazeau, Kelso and Prichard. We have:

1. Developed a new way to quantitate intermittent, bursting behavior from time series of interevent intervals called *Bernoulli partition equivalence*. We use the Erdos-Renyi theorem that in a binary game, maximum run length grows  $\approx \log (\text{base } 1/p) n$ . The partition of the normalized range of interspike intervals into  $\{0,1\}$  such that runs grow as  $\log n$  discriminates between classes of biogenic amine neurons.
2. Developed a new measure called the *nonuniformity of divergence*, which is the distribution and higher moments of the leading Lyapounov exponent and which successfully discriminates between biogenic amine neurons even though the mean values of the characteristic exponents of the time series of interspike intervals were not discriminable.
3. Showed that the characteristic power spectral transformations of the interspike interval series of biogenic amine neurons were not discriminable from the circle map at the critical line, ( $k = 1$ ) as first reported by Ostland, Rand, Siggia and Shenker and Kadanoff. They are also like the spectra of *strange nonchaotic attractors* which result from quasiperiodic driving of nonlinear systems in at least co-dimension two.
4. Used an piece-wise, convex, intermittency map, mod one, as a model of quasiperiodic rotations on the circle near a single homoclinic tangency to drive a reduced, discrete cubic map as a Hodgkin-Huxley, global membrane model demonstrating quasiperiodic driving and strange, nonchaotic attractor (Grebogi) as the EEG.
5. In the context of brain stem neuronal quasiperiodic driving of the EEG and the hypothesized resulting dynamical structure, we have noted the expected "period-adding" mode structure in this regime between quasiperiodicity and chaos, of the human EEG:  $\Delta(2-3 \text{ Hz}) + \theta(5-7 \text{ Hz}) =$

$\alpha$ (10-12 Hz);  $\theta + \alpha = \beta$ (20-24 Hz);  $\alpha + \beta = \gamma$ (35-50 Hz). We have applied the generalized Kolmogorov-Arnold-Moser arguments mode stability of the torus near breakdown and recent Crawford-Knowlton spatial mode symmetry arguments for the stability of nongeneric bifurcations and eigenvalue degeneracy in order to defend this conjecture. In addition, we have found support in EEG data from the work of Friedrich, Fuchs, and Haken who used the Karhunen-Loeve spatial mode decomposition which demonstrates the symmetry stability arguments directly.

**SIGNIFICANCE:** We have reinterpreted the "chaos theory of the EEG" and the dimension (of 6 to 7) as promulgated by over 30 laboratories world wide as a strange, nonchaotic theory involving quasiperiodic and intermittent driving of the EEG nonlinear oscillator as *quasiperiodic resonance of a strange, nonchaotic attractor* with 6 to 8 modes. *We speculate this involves the single neuron computation of global brain states as seen in stages of sleep and arousal.*

**WORKPLAN:** (1) Extend our work to reticular formation neurons; (2) Extend our work to time series of biogenic amine enzyme behavior regulating single neurons; (3) Extend our work to single pituitary cell hormone release dynamics as a slow neuron model of intermittency; (4) Study quasiperiodic resonance and strange nonchaotic attractors using time series taken from our EAI-680 analog computer with a variety of driving dynamics; (5) Continue to evolve statistical measures for these *strange nonchaotic dynamical behavior of single neurons and their models.*

**PUBLICATIONS:** (1) Paulus, M.P., Geyer, M.A., Gold, L.H., and Mandell, A.J. (1990) Application of entropy measures derived from the ergodic theory of dynamical systems to rat locomotor behavior. *Proc. Nat. Acad. Sci.* 87: 723-727. (2) Paulus, M.P., Geyer, M.A., and Mandell, A.J. (1991). Statistical mechanics of a neurobiological dynamical system: the spectrum of local entropies  $S(\alpha)$  applied to cocaine-perturbed behavior. *Physica A*. Aug. (3) Selz K.A. and Mandell, A.J. (1991) The Bernoulli partition equivalence of intermittent neuronal discharge patterns. *Int. J. Bifurc. Chaos* 1(3) Sept. (4) Selz, K.A. and Mandell, A.J. (1991). Critical coherence and characteristic times in brain stem neuronal discharge patterns. In *Single Neuron Computation* (Ed. McKenna, Davis, and Zornetzer). Academic Press. N.Y. (5) Mandell, A.J. (1991). Is the EEG a strange attractor? Brain stem neuronal discharge patterns and electroencephalic rhythms. In *The Impact of Chaos on Science and Society*. (Ed. Grebogi and Yorke). United Nations Press. Japan. (6) Mandell, A.J. and Shlesinger, M.F. (1990). Lost choices: parallelism and topological entropy decrements in neurobiological aging. In *The Ubiquity of Chaos* (Ed. Krasner) AAAS Press. pp 35-46; (7) Mandell, A.J. (1990) Parameter-induced, high period mode locking and nonuniform quasiperiodic bursting scenarios in sleep stage transitions in *Endogenous Sleep Factors* (Ed. Inoue and Krueger). SPB Academic, Netherlands, pp 307-321 (8) Selz, K.A. and Mandell, A.J. (1990) Heterochrony as a generalizable principle in biological dynamics. In *Propagation of Correlations in Constrained Systems* (Ed. Stanley and Ostrowsky). Plenum 280-292; (9) Kelleher, J.F. and Mandell, A.J. (1990). Dystonia musculorum deformans as a dopaminergic critical phenomena in *Medical Hypotheses* 31: 55-58. (10) Mandell, A.J. and Selz, K.A. (1991). A nonthermodynamics formalism for biological information systems. in *Self-Organization, Emerging Properties, and Learning*. (Ed. Babloyantz) NATO. Plenum. N.Y.

## ANNUAL PROGRESS REPORT

GRANT #: N00014-90-J-1865

R&T CODE: 4414029

PRINCIPAL INVESTIGATOR: Mu-ming Poo

INSTITUTION: Columbia University

GRANT TITLE: Electrokinetic Modulation of Synaptic Efficacy

REPORTING PERIOD: 1 June 1990 - 31 May, 1991 (12 months)

AWARD PERIOD: 1 June 1990 - 31 May, 1993

OBJECTIVE: To study the effect of electrical activity on the efficacy of synaptic transmission in model cell culture system, and to test the hypothesis that direct electrokinetic actions of the synaptic current may be responsible for the activity-dependent modulation of synaptic structure and function.

ACCOMPLISHMENTS (last 12 months): We have developed a nerve-muscle culture in which a single muscle cell was co-innervated by two embryonic spinal neurones, and the effect of electrical activity on the synaptic efficacy was examined after electrical stimulation was applied to one or both neurons. We found that brief tetanic stimulation of one neuron (50-100 pulses at 2-5 Hz) results in immediate functional suppression of the synapse made by the other unstimulated neuron co-innervating the same muscle cells, as shown by a reduction in the amplitude of impulse-evoked synaptic currents recorded in the myocyte. Heterosynaptic suppression was observed regardless whether the tetanized synapse showed stronger or weaker synaptic efficacy prior to the tetanus, although stronger synapses were found to be more effective in inducing suppression. Synchronous application of tetanic stimulation to both neurons resulted in little or no suppression of both synapses, while the same tetani applied asynchronously (with 250 ms delay) to the two neurons led to marked suppression of one or both synapses. Finally, the heterosynaptic suppression was found to be absent when the distance between the sites of innervation made by two neurons was larger than 50-75  $\mu\text{m}$ , suggesting competitive interaction underlying the heterosynaptic suppression occurs only among adjacent synapses.

SIGNIFICANCE: These results represent the first in vitro demonstration of Hebbian modulation of synaptic efficacy. It may allow fruitful investigation of the cellular and molecular mechanisms underlying activity-dependent synaptic modulation.

WORK PLAN (Next 12 months): We will continue our study of the synaptic competition in culture, with main emphasis on the understanding of the cellular mechanism underlying the heterosynaptic suppression. We hope to determine whether the suppression involves direct interactions between pre-synaptic nerve terminals or is mediated by postsynaptic activation of the muscle cells. We will further examine whether the distribution of membrane and cytoplasmic components in the muscle cell is affected by the tetanic stimulation; if so, whether the redistribution is causally related to changes in synaptic efficacy.

PUBLICATIONS AND ABSTRACTS (supported by the present award)

1. Lo, Y., T. Wang and M-m. Poo. (1991) Repetitive impulse activity potentiates spontaneous acetylcholine secretion at developing neuromuscular synapses. J. Physiol. (Paris), in press.
2. Lo, Y. and M-m. Poo. (1991) Heterosynaptic suppression of developing neuromuscular synapses: Hebbian Modulation in vitro. Soc. Neurosci. Abs. In press.
3. Popov, S. and M-m. Poo (1991) Diffusional transport of macromolecules in developing nerve processes. Soc. Neurosci. Abs. In press.
4. Dan, Y. and M-m. Poo (1991) Suppression of developing neuromuscular synapses by focal iontophoretic application of acetylcholine. Soc. Neurosci. Abs. In press.

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